FOLIC ACID DEFICIENCY AND URINARY FORMIMINOGLUTAMIC ACID EXCRETION IN THE RAT

by R. E. DAVIS

(From the Department of Haematology, Royal Perth Hospital, Western Australia).

(*Accepted for publication 7th August, 1961.*)

SUMMARY.

Rats fed on a folic acid-deficient diet regularly excrete formiminoglutamic acid in the urine. The vitamin B_{12} and folic acid status of such rats was estimated by assay of liver tissue for these vitamins. Formiminoglutamic acid excretion occurs in the presence of normal liver vitamin B_{12} concentrations. There appears to be a critical level of liver folic acid below which there is excretion of formiminoglutamic acid.

INTRODUCTION.

Formiminoglutamic acid is an intermediate product in the breakdown of histidine and its further metabolism requires tetrahydrofolic acid (Tabor and Wyngarden, 1958). It appears in the urine of rats made folic acid deficient (Rabinowitz and Tabor, 1958). The present investigation was designed to assess any correlation between the degree of folic acid deficiency and the quantitative excretion of formiminoglutamic acid in the rat.

METHODS.

Three groups of 10 Wistar rats were fed on controlled diets from the time of weaning. Group 1 was given a standard cubed diet (see appendix) which is considered to be nutritionally adequate for normal growth and development. Group 2 was fed a synthetic diet deficient in folic acid (see Appendix) but supplemented with folic acid (20 mg. weekly) added to the drinking water. Group 3 received the folic acid-deficient diet without the folic acid supplement. Animals in all three groups received vitamin B_{12} (2 μg. weekly) added to the drinking water. When aged 9 months each rat was held in a metabolic cage for 48 hours and the urine collected. The rats were then killed and the livers assayed for vitamin B_{12} and folic acid.

All specimens of urine were adjusted to pH 4·0 and stored at 4° C. until required. Urinary formiminoglutamic acid was assayed quantitatively by a microbiological method using *L. arabinosus* as test organism (Davis and Onesti, 1960). Liver folic acid was estimated after papain digestion (Pitney and Onesti, 1961) using a mutant strain of *Streptococcus lactis R* (ATCC 8043). Liver vitamin B_{12} was assayed with the Z strain of *Euglena gracilis* (Hutner, Bach and Ross, 1956).

RESULTS.

Two rats in Group 3 died during the course of the experiment and have been excluded from the results. Both died from bacterial infection. Table 1 shows the results obtained from the 10 rats in Group 1 fed a normal diet with vitamin B_{12} supplement. The weight of these rats at 9 months ranged from 116 gm. to 270 gm. (mean 206 gm.). The total vitamin B_{12} in the livers ranged from 0·38 μg. to 0·73 μg. (mean 0·56 μg.) and total folic acid from 7·24 μg. to 36·01 μg. (mean 17·53 μg.). Values are expressed as total amounts in the organ rather than as concentrations to obviate the influence of varying liver weights. One animal in the control group excreted a trace of formiminoglutamic acid in the urine. This was insufficient for quantitative assessment.

Table 2 shows the results in the 10 rats in Group 2 (folic acid-deficient diet with folic acid and vitamin B_{12} supplements). Total body weights ranged from 114 gm. to 203 gm. (mean 150 gm.). Total liver B_{12} ranged from 0·37 μg. to 0·84 μg. (mean 0·66 μg.) and total folic acid from 6·70 μg. to 27·77 μg. (mean 14·05 μg.). No animal excreted formiminoglutamic acid in the urine.

Table 3 shows the results in the eight surviving Group 3 rats (folic acid-deficient diet with vitamin B_{12} supplement). Total body weights ranged from 126 gm. to 212 gm. (mean 164 gm.). Total liver B_{12} ranged from 0·61 μg. to 1·07 μg. (mean 0·77 μg.) and total folic acid from 2·08 μg. to 4·50 μg. (mean 3·20 μg.). Seven rats excreted measurable amounts of formiminoglutamic acid in the urine. These values are given as concentrations rather than as 48-hour total excretions as it was not found possible to collect all urine passed by these animals.

TABLE 1.

Rats fed a normal diet with vitamin B_{12} supplement (Group 1).

Key	Body weight (gm.)	Liver weight (gm.)	Total liver B_{12} (μg.)	Total liver folic acid (μg.)	FGA* excretion
A1	116	4·17	0·38	21·14	0
A2	258	8·15	0·57	12·39	0
A3	147	5·24	0·73	7·65	0
A4	211	5·94	0·53	7·24	0
A5	154	4·33	0·61	17·15	0
A6	270	8·13	0·65	20·49	0
A7	232	8·53	0·60	13·90	trace
A8	246	7·94	0·48	10·88	0
A9	191	5·99	0·48	28·45	0
A10	240	5·78	0·52	36·01	0

* Formiminoglutamic acid.

TABLE 2.

Rats fed a folic acid-deficient diet with folic acid and vitamin B_{12} supplement (Group 2).

Key	Body weight (gm.)	Liver weight (gm.)	Total liver B_{12} (μg.)	Total liver folic acid (μg.)	FGA excretion
B1	134	5·36	0·75	6·70	0
B2	148	6·58	0·66	8·69	0
B3	114	4·54	0·68	12·67	0
B4	124	4·88	0·68	11·18	0
B5	150	6·63	0·66	11·54	0
B6	193	8·02	0·56	10·83	0
B7	134	4·48	0·72	19·58	0
B8	176	5·25	0·84	27·77	0
B9	128	5·30	0·37	13·30	0
B10	203	8·55	0·68	18·21	0

TABLE 3·

Rats fed a folic acid-deficient diet with vitamin B_{12} supplement (Group 3).

Key	Body weight (gm.)	Liver weight (gm.)	Total liver B_{12} (μg.)	Total liver folic acid (μg.)	FGA excretion (μg./ml.)
C1	126	7·65	1·07	3·44	110
C2	162	6·61	0·66	2·25	250
C3	212	7·19	0·86	4·10	0
C4	140	6·11	0·79	2·08	160
C5	210	8·29	0·75	4·50	75
C6	176	6·94	0·63	3·50	300
C7	138	5·44	0·76	2·90	310
C8	148	6·11	0·61	2·90	98

DISCUSSION.

These results confirm previous observations that folic acid-deficient rats regularly excrete formiminoglutamic acid in the urine. In the present experiment folic acid deficiency was achieved without the administration of succinyl-

sulphathiazole with the folic acid-deficient diet. Sulphonamide administration, by altering the bacterial population in the gut, may produce a more profound degree of folic acid deficiency but such animals tend to develop diarrhoea and mortality is high. Sulphonamide administration is undesirable in experiments involving microbiological assays of tissues and urine since growth inhibition of the test organism may be produced unless p-aminobenzoic acid is added to culture media.

The mean body weights of Group 2 and Group 3 rats were similar and were less than that of Group 1 rats. This observation suggests that the folic acid-deficient diet was not entirely adequate in other respects since Group 2 received folic acid supplementation. It suggests, also, that growth failure on a folic acid-deficient diet may be due to deficiencies other than folic acid.

Total liver B_{12} content was similar in all groups of animals and the values in each group fell into a rather narrow range. This observation suggests that folic acid deficiency does not influence the liver B_{12} content provided that B_{12} intake is adequate.

Liver folic acid values were scattered over a wide range in Group 1 and Group 2 animals. Mean values were comparable, however (17·53 μg. and 14·05 μg. respectively). Both values are considerably higher than the mean value of 3·20 μg. obtained with Group 3 (folic acid-deficient) animals. The presence of demonstrable liver folic acid in Group 3 suggests that formiminoglutamic acid excretion occurs before there is complete depletion of folic acid in the liver. An alternative explanation is that some material assayed as folic acid may not have biological importance in the further metabolism of formiminoglutamic acid.

The results show a correlation between liver folic acid content and the appearance of urinary formiminoglutamic acid. In Groups 1 and 2, the lowest liver folic acid values were 7·24 μg. and 6·70 μg. respectively. These animals did not excrete formiminoglutamic acid. The highest liver folic acid value in Group 3 was 4·50 μg. and seven out of eight rats in this group excreted formiminoglutamic acid. Furthermore, the two highest readings in this group were associated with the least amounts of formiminoglutamic acid (rats C3 and C5).

Acknowledgments. The author wishes to thank Dr. W. R. Pitney for advice and criticism, Dr. Barbara J. Arnold for help with the folic acid-deficient diets, and Mr. A. Kelly for the vitamin B_{12} and folic acid assays.

REFERENCES.

Davis, R. E., and Onesti, P. (1960): Austral. J. exp. Biol., 38, p. 239.
Hutner, S. H., Bach, M. K., and Ross, G. I. M. (1956): J. Protozool., 3, p. 101.
Pitney, W. R., and Onesti, P. (1961): Austral. J. exp. Biol., 39, p. 1.
Rabinowitz, J. C., and Tabor, H. (1958): J. biol. Chem., 233, p. 252.
Tabor, H., and Wyngarden, L. (1958): J. clin. Invest., 37, p. 824.

APPENDIX.

Formula of standard cubed diet (obtainable from Wesfarmers Pty. Ltd., Perth, W.A.):

Ground wheat	50 p.c.	Brewers' yeast	5 p.c.
Barley meal	12½ p.c.	Dried skim milk	5 p.c.
Lucerne meal	5 p.c.	Oyster flour	½ p.c.
Whale meal	16 p.c.	Molasses	3 p.c.
Bone meal	2 p.c.	Sodium chloride	½ p.c.

Riboflavin	3 mg. per Kg.
Vitamin A	8,000 I.U. per Kg.
Vitamin D	1,000 I.U. per Kg.

Formula of folic acid-deficient diet:

Sucrose	710 gm.		
Lactic casein	180 gm.		
Peanut oil	50 gm.		
Ferric citrate $5H_2O$	10 gm.		
Choline chloride	2 gm.		
Copper sulphate	0·4 gm.		
ABDEC (Parke Davis)	10 ml.		
Biotin	0·01 mg.		
Vitamin K	5 mg.		
Salt mixture	40 gm.		

Salt mixture (all anhydrous salts):

Calcium carbonate	134·8 gm.
Magnesium carbonate	24·2 gm.
Sodium carbonate	34·2 gm.
Potassium carbonate	141·3 gm.
Phosphoric acid	103·2 gm.
Hydrochloric acid	53·4 gm.
Sulphuric acid	9·2 gm.
Citric acid	111·1 gm.
Potassium iodide	0·020 gm.
Manganese sulphate	0·079 gm.
Sodium fluoride	0·248 gm.
Potassium aluminium sulphate	0·245 gm.

Mix acids and add carbonates. Trace elements are added as solutions of known concentrations. Evaporate to dryness at 90°-100° C. and grind to fine powder.

A COMPARATIVE STUDY OF BLOOD COAGULATION IN VERTEBRATES

by P. FANTL

(From the Baker Medical Research Institute, Melbourne).

(*Accepted for publication 2nd August, 1961.*)

SUMMARY.

The addition of kaolin suspension and phospholipid shortens the clotting time of plasma of toad, trout, echidna, tortoise, possum, dog, guinea pig, rat, man and lizard. Kaolin has little influence on the plasma clotting time of the domestic fowl, teal and tiger snake. The duration of incubation of plasma with kaolin suspension and phospholipid to produce shortest clotting time varies from less than one minute in the plasma of the first four vertebrates to several minutes in the plasma of lizards and man. The kaolin-activated clotting factors are very unstable in plasma of toad, trout, tortoise and echidna and stable for several minutes in the plasma of the other vertebrates. The addition of bird or snake plasma does not correct the coagulation defect of human plasma congenitally deficient in Hageman factor. Toad plasma has a low activity in this respect. Thromboplastin formation takes place in all the examined plasma but is low, in decreasing order, in the plasma of lizard, tortoise and tiger snake.

In the conversion of prothrombin homologous lung suspensions are usually more active than heterologous ones. Clotting times obtained in the presence of Russell's viper venom or trypsin indicate a low prothrombin activity in the plasma of the tiger snake.

In the plasma of the tiger snake the rate of reaction of thrombin with fibrinogen and the fibrinogen concentration are adequate for haemostasis.

INTRODUCTION.

It has long been known that the clotting time of mammalian blood is shorter in hydrophilic containers, e.g., glass, than in hydrophobic containers, e.g., silicones. This dependence on surface properties has been recently recognised as due to two plasma components, namely, the Hageman factor and the plasma thromboplastin antecedent (P.T.A.).

Recently we examined an 11-year-old boy with Ehlers-Danlos syndrome whose blood did not clot normally *in vitro* because of the absence of Hageman factor from his plasma. However, his defects did not result in excessive bleeding after cardiac surgery (Fantl, Morris and Sawers, 1961). Ratnoff and Margolius (1957) found that patients with the Hageman trait do not bleed excessively even after severe trauma. The only demonstrable change in these patients is altered tissue permeability (Margolis, 1959).

Because of the lack of readily recognisable symptoms a deficiency of Hageman factor is detected only by chance and the frequency of the deficiency in human blood is therefore unknown. However, it has been reported that this defect is characteristic of the blood of birds (Ratnoff and Margolius, 1957; Wartelle, 1957; Didisheim *et al.*, 1959; and Thompson *et al.*, 1960). For the study of the rôle of Hageman factor in biological processes a source of plasma with this deficiency would be useful so, in view of the report of its deficiency in the blood of birds, it was of interest to compare the coagulation activity of the blood of a number of vertebrates.

Materials and Methods.

The vertebrates examined were as follows and the number of specimens tested is given in parenthesis: fresh water trout, *Salmo trutta*, from the Snobs Creek hatchery of the Victorian Department of Fisheries and Wildlife (4); cane toad, *Bufo marinus*, from Queensland (12 pooled from 26 toads); long-necked tortoise, *Chelodina longicollis*, from the Murray River at Tocumwal in New South Wales (6); blue tongue lizards, *Tiliqua scindoides*, from the Werribee plains, Victoria (9); shingle back lizards, *Tiliqua rugosa*, from the Eyre Peninsula, South Australia (4); tiger snakes, *Notechis scutatus*, from Tocumwal, New South Wales (9); echidnas, *Tachyglossus aculeata*, from Healesville, Victoria (3). Possums, *Trichosurus vulpecula*, were trapped in Melbourne (2); domestic fowl were bought on the local market (5).

Trout, lizards and snakes were stunned by a blow on the head; toads and tortoises were pithed. The heart was exposed and one branch of the aorta was clamped and the other punctured with a needle. Tiger snakes were bled from the dorsal aorta. Other species were anaesthetised (echidna, possum and domestic fowl with a mixture of ether and oxygen, dogs and rats with nembutal) and bled from the jugular vein. All animals were in a good nutritional condition. Human blood was taken from the cubital vein.

One volume of 0·13 M trisodium citrate and nine volumes of blood were mixed. The blood specimens were chilled to 2° C., centrifuged in the cold and the cell-free plasma kept cold until the determinations were carried out not more than six hours after collection.

Lungs were perfused with Ringer solution until free from blood and were then dried by blotting. The tissue was cut into small pieces, mixed with 2 parts of 0·154 M NaCl and incubated, with frequent maceration, at 35° C. for two hours. The mixture was allowed to stand for half an hour and the supernatant suspension, free of large particles, was pipetted off before use.

Russell's viper venom (Ross Allen, Silver Springs, Florida, U.S.A.) was dissolved in 0·154 M NaCl to a concentration of 0·1 mg./ml. Trypsin ("Trypure", Novo Copenhagen) was dissolved in 0·154 M NaCl. Phospholipid was prepared according to Fantl (1958). Kaolin (B.D.H. Society of Leather Trades Chemists' specification) was suspended in 0·154 M NaCl to a concentration of 100 mg./ml. Bovine thrombin (Parke Davis) was used.

RESULTS.

Hageman factor.

As the Hageman factor is activated when blood is in contact with certain surfaces (Margolis, 1958) the rate of activation of plasma was determined by the following procedure: 0·2 ml. of citrated plasma was incubated at 35° C. with 0·1 ml. of a suspension containing 30 μg. phospholipid and 0·05 ml. of a

10 p.c. kaolin suspension. After incubation for the times indicated in Figs. 1 and 2, 0·2 ml. of 0·025 M $CaCl_2$ was added quickly and the clotting time determined. The results are given in Figs. 1 and 2.

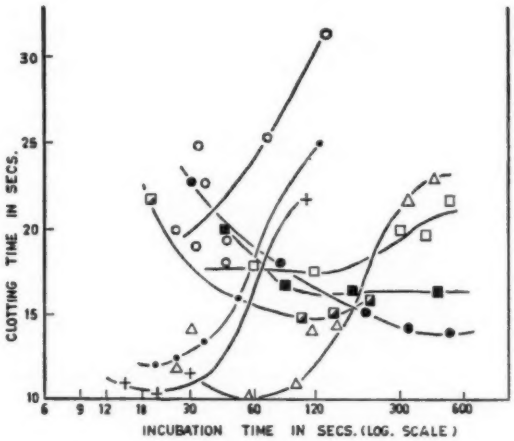

Fig. 1. Plasma of: +—toad, ●—trout, △—echidna, ○—tortoise, ◪—possum, ●—dog, ■—guinea pig, □—rat.

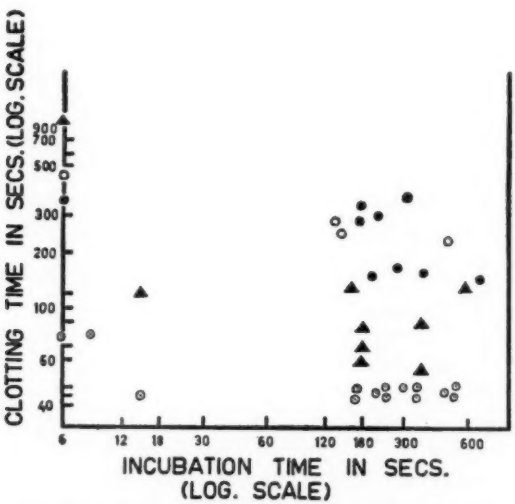

Fig. 2. Plasma of: ⊙—man, ▲—lizards, ○—domestic fowl, teal, ●—tiger snake.

It is seen from Figs. 1 and 2 that clotting times of the plasma increased in the following order: toad, trout, tortoise, echidna, other mammals, lizard, birds (domestic fowl, teal) and tiger snake. In the case of the plasma of toad, trout, tortoise and echidna the shortest clotting times were obtained after incubation with kaolin and phospholipid for approximately 30 seconds. With more prolonged incubations the clotting times became progressively longer. In the case of the plasma of the other vertebrates the shortest clotting time was obtained after incubation with the kaolin and phospholipid suspension for more than one minute but in contrast to the aforementioned species the clotting time remained constant for incubation times of several minutes. Treatment with kaolin suspension had little influence on the plasma clotting time of birds and snakes. A comparison between the clotting times in the absence and presence of kaolin is given in Table 1.

The results in Table 1 indicate that in the absence of kaolin the clotting times are in general longer than in its presence, but the order of activity is the same as that in Figs. 1 and 2.

Direct evidence of Hageman factor activity was obtained by the addition of plasma to human plasma congenitally deficient in Hageman factor. Results are given in Table 2.

TABLE 1.

Influence of kaolin suspension on plasma clotting time.

To 0·2 ml. citrated plasma was added 0·1 ml. phospholipid suspension in all cases. In A 0·05 ml. 0·154 M NaCl and 0·2 ml. 0·025 M $CaCl_2$ were added. In B a mixture of 0·05 ml. 10 p.c. kaolin suspension in 0·154 M NaCl and 0·2 ml. 0·025 M $CaCl_2$ were added. In C 0·05 ml. 10 p.c. kaolin suspension was added and after incubation 0·2 ml. 0·025 M $CaCl_2$ was added at 35° C. In this and following Tables the average clotting times are given in seconds: the range of values is shown in brackets.

Plasma of	A In absence of kaolin	B In presence of kaolin without incubation	C After incubation with kaolin	Incubation (min.)
Toad	73 (55-100)	20 (15-27)	11·5 (6-15)	0·25
Trout	54 (27-82)	18 (14-23)	12	0·25
Echidna	27 (17-45)	20 (15-25)	12 (10-14)	0·3
Tortoise	72 (40-160)	31 (29-34)	26 (19-39)	0·5-1
Dog, guinea pig and possum	30-59	—	15-21	1·5-3
Man	82 (70-90)	70 (62-81)	44 (40-57)	3
Lizard	1285 (400-2000)	506 (115-930)	111 (70-257)	3-14
Domestic fowl	390 (318-946)	—	206 ± 50	4-12
Tiger snake	367 (258-489)	301 (135-489)	224 (92-290)	3-10

TABLE 2.

Influence of normal plasma on coagulation of human plasma congenitally deficient in Hageman factor.

	Addition of plasma in ml.*	Clotting time (sec.)
(1) Human Hageman factor deficient plasma	0	520-620
(1) plus normal human plasma	0·01	100
(1) plus toad plasma	0·01	180
(1) plus domestic fowl plasma	0·02	600
(1) plus tiger snake plasma	0·05	320-460

* To 0·2 ml. human Hageman factor-deficient plasma were added 0·1 ml. phospholipid suspension, the volume of plasma as indicated and 0·2 ml. 0·025 M calcium chloride, 35° C.

In addition to the experiments given in Table 2 in another series 0·02 ml. plasma was added to clotting tubes and after contact for 15 minutes at 2° C. the non-adsorbed plasma was removed by repeated rinsing with 0·154 M NaCl. Hardisty and Margolis (1959) have shown that the film adsorbed by glass has Hageman factor activity. To the pretreated clotting tubes the other additions as in Table 2 were made. The results were similar to those tabulated.

The figures in Table 2 are probably only of a qualitative nature because of a possible species specificity of Hageman factor. However, they indicate that addition of 5 p.c. of normal human plasma corrects the Hageman factor deficiency. Toad plasma is far less effective and the plasma of domestic fowl and of the tiger snake have very little influence.

It appears that the plasma of the examined vertebrates, with the exception of that of birds and the tiger snake, contain Hageman factor.

The results shown in Figs. 1 and 2 and Tables 1 and 2 depend on the activity of the components of the thromboplastin complex, the prothrombin complex and on fibrinogen, leaving inhibitors out of consideration.

Thromboplastin and thrombin.

In the following experiments the ability of various plasma to produce thromboplastin and thrombin was determined. Thromboplastin formation was measured by incubation of citrated plasma with phospholipid and calcium ions. Aliquots were added to recalcified citrated plasma. The clotting times are given in Table 3.

These results show that with regard to the ability to form plasma thromboplastin little differentiation can be made between the plasma of mammals and that of the trout and the toad, but the plasma of lizard, tortoise and tiger snake show reduced thromboplastin formation.

TABLE 3.

Thromboplastin formation in vertebrate plasma.

0·12 ml. citrated plasma and 0·6 ml. phospholipid suspension, 0·6 ml. veronal buffer pH 7·4, 0·6 ml. 0·025 M $CaCl_2$ and 1·08 ml. 0·154 M NaCl were incubated at 28° C. 0·2 ml. was added to 0·2 ml. citrated human plasma followed by 0·2 ml. 0·025 M $CaCl_2$. The shortest clotting times are recorded. In some cases homologous plasma was used as substrate.

Citrated plasma of	Clotting time (sec.)	Incubation time (min.)
Trout	7-9	2
Man	8·5-10	3-5
Toad	10	2
Echidna	8·5	<2
Possum	11	2
Lizard*	13 (11-17)	8-15
Tortoise	18 (16-22)	4
Tiger snake	24 (21-28)	4-14

* Fed lizards (egg yolk and milk); fasting lizards showed longer clotting times.

Prothrombin complex.

In further experiments the activity of the prothrombin complex was determined. Lung suspension and calcium ions were added to citrated plasma. The results are shown in Table 4.

It is seen from Table 4 that all the lung suspensions, with the exception of that of the tiger snake, gave the shortest clotting time with homologous plasma. There are peculiar relations between the source of lung and clotting time. Whereas lizard lung gave practically identical clotting times with lizard and toad plasma, toad lung showed a distinct species specificity. Suspensions of the lung of the tiger snake gave short clotting times with homologous and also with lizard plasma. Lizard lung showed hardly any activity in the conversion of prothrombin of the tiger snake. Lung suspensions, with the exception of that of the tiger snake, gave with homologous plasma clotting times of 11-13 seconds. This is similar to clotting times one obtains with homologous brain extract on human plasma.

TABLE 4.

Influence of lung suspension on plasma clotting time (secs.).

To 0·1 ml. of citrated plasma were added 0·2 ml. of a mixture of equal volumes of lung suspension and 0·025 M calcium chloride at 35° C.

	Lung suspension of			
Citrated plasma of	Lizard	Toad	Tortoise	Tiger snake
Man	120 (107-145)	141 (132-159)	105 (95-116)	98 (63-124)
Lizard	12·5 (9·5-16·5)	182 (50-315)	20·5	22 (15-26)
Toad	12·5 (12-13·5)	11 (8-13)	—	—
Tortoise	78 (37-110)	—	13·5 (12-16·5)	46 (30-76)
Tiger snake	620 (380-960)	580	173 (111-229)	35 (26-42)
Trout	66	90	—	—

In the case of mammalian plasma the clotting time in the presence of an homologous organ extract depends on the activity of prothrombin and factors V, VII, X, and fibrinogen. A reduction of any one of them prolongs the clotting time. If organ suspensions are replaced by Russell's viper venom the results given in Table 5 are obtained.

TABLE 5.

Influence of Russell's viper venom and phospholipid on plasma clotting time (secs.).

To 0·1 ml. citrated plasma were added 0·1 ml. phospholipid suspension, 0·05 ml. Russell's viper venom in 0·154 M NaCl and 0·1 ml. 0·025 M $CaCl_2$ at 35° C. The various plasma specimens were obtained at different times and did not include all the species given in the Table, but in each series human plasma was used.

Citrated plasma of	Clotting time
Mammals	7·5 (6·5-9)
Lizard	8 (6·5-11)
Toad	9·5 (6·5-14)
Tortoise	10 (8-11·5)
Domestic fowl	14 (11·5-16)
Trout	13 (12-18)
Tiger snake	24 (16-38)

The results in Table 5 show that the plasma of mammals, lizard, toad and tortoise show little difference in clotting time, but the plasma of domestic fowl, trout, and especially of the tiger snake give considerably longer clotting times.

The clotting time of plasma in the presence of Russell's viper venom, phospholipid, and calcium ions depends upon the activity of prothrombin, factors V and X, and on fibrinogen concentration. The results in Tables 4 and 5 indicate that most of the examined plasma contained adequate concentration of prothrombin and factors V, VII and X. The plasma of the tiger snake is a notable exception. In order to obtain more information on the activity of plasma prothrombin, clotting times in the presence of trypsin were determined, because trypsin converts prothrombin into thrombin in the absence of other clotting factors.

TABLE 6.

Influence of trypsin and phospholipid on plasma clotting time (secs.).

To 0·2 ml. citrated plasma were added 0·1 ml. phospholipid suspension, 0·05 ml. trypsin solution containing 0·05 mg. trypure and 0·2 ml. 0·025 M $CaCl_2$ at 35° C.

Citrated plasma of	Clotting time
Trout	8·5 (8-9)
Toad	11·5 (10·5-12)
Tortoise	11 (9-12)
Man	14 (11-16)
Lizard	17·5 (15-20)
Domestic fowl	23 (19-26)
Tiger snake	34 (22-49)

The order of activity seen in Table 6 is not the same as that in Table 5. The clotting time of the trout plasma is shorter in the presence of trypsin than in the presence of Russell's viper venom. In both instances the plasma of the tiger snake gives considerably longer clotting times than any other plasma. From these results it appears that tiger snake plasma has a lower prothrombin activity than other vertebrates.

Fibrinogen.

Finally, the clotting time depends on the rate of the fibrinogen-thrombin reaction, indicated by the thrombin clotting time and the fibrinogen concentration. Thrombin clotting times are given in Table 7. It is seen from the results

TABLE 7.

The thrombin clotting time of vertebrate plasma.

To 0·2 ml. citrated plasma 0·1 ml. bovine thrombin in 0·154 M NaCl was added. The clotting times with human plasma varied between 12 and 15 secs. As human plasma gave in each case the shortest clotting time its activity was designated as 100. The activity of the other plasma was determined from a curve relating clotting time to thrombin activity. (Fantl, 1954).

Citrated plasma of	Activity
Man	100
Trout	80
Tiger snake	66 (57-80)
Toad	28 (15-50)
Lizard	25 (10-50)
Echidna	20
Tortoise	12 (10-13)

in Table 7 that bovine thrombin gave shortest clotting time with human plasma. In contrast to its behaviour in other clotting tests the plasma of tiger snake showed considerable reactivity in the thrombin-fibrinogen reaction.

Chemical determination of fibrinogen in the various plasma gave the following values (in gm. per 100 ml.): man, 0·2-0·4; trout, 0·25; tiger snake, 0·14; lizard, 0·14.

DISCUSSION.

The above results indicate that clotting factors which can be activated by glass and kaolin occur in plasma of the vertebrates investigated with the exceptions of birds and the tiger snake. In addition to Hageman factor plasma thromboplastin antecedent (P.T.A.) is involved in contact activation but as P.T.A.-deficient plasma was not available no statement regarding this clotting factor can be made. Hageman factor activity was tested by the ability of the plasma to influence the clotting of a human plasma deficient in Hageman factor. It was shown that bird and snake plasma did not correct the deficiency. However, it should be pointed out that species specificity of the plasma factor has not been investigated.

The stability of the kaolin-activated clotting factors is a characteristic feature of any particular plasma. Most vertebrate plasma can form thromboplastin of similar high activity, but those of the lizard, tortoise, birds and the tiger snake have low activity.

With regard to thrombin formation the combination of plasma and lung suspension showed no absolute species specificity, although a markedly higher activity in the homologous system was apparent. The results obtained with toad lung and plasma are in agreement with those given by Hackett and Le Page (1961). An exception was the combination of lung suspension and plasma of the tiger snake.

In the presence of Russell's viper venom the plasma of birds and of the tiger snake showed delayed clotting when compared with the other vertebrate plasma.

The activation of prothrombin by trypsin indicated a very low activity of prothrombin in the plasma of the tiger snake. On the other hand, the clotting time with bovine thrombin and the amount of fibrinogen in plasma of the tiger snake was not abnormally low. The thrombin-fibrinogen reaction, as previously noted (Fantl and Ward, 1957), was again fastest with human plasma.

The physiological or pathological significance of the blood clotting factors has been derived mainly from experience with mammals. Sufficient information is available to correlate activity as determined by laboratory tests with a haemorrhagic condition in the case of deficiencies, or with thrombosis in the case of increased activities. Applying the same criteria to other vertebrates both extremes in clotting activity are seen in the investigated plasma. Toads and trout have a very active clotting process which is contrasted with the sluggish process in birds and snakes.

Coagulation is a temperature-dependent process and Anstall and Huntsman (1960) have pointed out that the clotting mechanism of the toad is well adapted to low temperatures. Wolf (1959) also finds and quotes other evidence for fast blood coagulation of fresh water bony fishes. He observed that, in contrast to the situation in mammalian red cells which haemolyse to yield clear aqueous solutions of haemoglobin, the plasma-free cells of trout blood very quickly formed a gelatinous material when mixed with water. All the evidence indicates that even at low temperatures the reduced clotting activity is still adequate for haemostasis in trout and toad. At the other extreme with a low clotting efficiency are birds and the tiger snake. Birds' blood is deficient in three plasma thromboplastin components, Hageman factor, P.T.A. and factor IX (Wartelle, 1957; Soulier *et al.*, 1959) yet we have not lost any birds from haemorrhage following trauma and surgery.

In contrast, it may be pointed out that in man even a moderate reduction of factor IX may lead to serious haemorrhages. Particularly puzzling is the extremely low activity of the blood clotting system of the tiger snake. The

relationship between the activity of a clotting factor as found in laboratory tests and clinical symptoms observed in mammals is apparently not applicable to birds and snakes.

It is possible that blood coagulation in the normal organism is unnecessary and even undesirable. However, following trauma in man a comparatively fast coagulation process is essential for efficient haemostasis. Birds and particularly snakes perhaps bypass the plasma coagulation system and rely on a mechanism of vaso-constriction and rapid liberation of tissue thromboplastin following trauma (Bigland and Triantaphyllopoulos, 1960). This gives adequate haemostasis without some components of the thromboplastin complex, namely, Hageman factor, P.T.A., factors VIII and IX. But the plasma of the tiger snake appears to be abnormally low also in prothrombin when compared to mammalian plasma. The only clotting factor which is present in adequate concentration in plasma of tiger snake is fibrinogen. Despite a serious clotting deficiency it does not appear that birds and snakes are at a disadvantage because of an inadequate plasma coagulation system. This poses the question whether the level of fibrinogen is the deciding factor in the haemostasis of birds and snakes.

Acknowledgments. Part of the expenses were covered by a grant from the National Health and Medical Research Council. The Department of Fisheries and Wild Life of Victoria has supplied the fresh water trout and has given permission to bleed echidna and possum. Mr. C. Tanner, Curator of Animal Husbandry, Alfred Hospital, Prahran, has provided the laboratory animals and collected the others. Miss Janet Howells has given technical assistance.

REFERENCES.

Anstall, H. B., and Huntsman, R. G. (1960): Nature, 186, p. 726.
Bigland, C. H., and Triantaphyllopoulos, D. C. (1960): *Ibid.*, 186, p. 644.
Diddisheim, P., Hattori, K., and Lewis, J. H. (1959): J. lab. clin. Med., 53, p. 866.
Fantl, P. (1954): Biochem. J., 57, p. 416.
Fantl, P. (1958): *Ibid.*, 36, p. 581.
Fantl, P., and Ward, H. A. (1957): Austral. J. exp. Biol., 35, p. 209.
Fantl, P., Morris, K., and Sawers, R. J. (1961): Brit. Med. J., I, p. 1202.
Hackett, E., and Le Page, R. (1961): Austral. J. exp. Biol., 39, p. 67.
Hardisty, R. M., and Margolis, J. (1959): Brit. J. Haematol., 5, p. 203.
Margolis, J. (1958): J. clin. Path., 11, p. 406.
Margolis, J. (1959): Austral. J. exp. Biol., 37, p. 239.
Ratnoff, O. D., and Margolius, A. (1957): Hemophilia and Hemophilioid Diseases. Editor—K. M. Brinkhous. Chapel Hill. University of North Carolina Press.
Soulier, J. P., Wartelle, O., and Ménaché, D. (1959): Brit. J. Haematol., 5, p. 121.
Thompson, J. H., Spittel, J. A., Pascuzzi, C. A., and Owen, C. A. (1960): Proc. Mayo Clinic, Vol. 35, p. 421.
Wartelle, O. (1957): Rev. D'Hématologie, 12, p. 351.
Wolf, K. (1959): Blood, 14, p. 1339.

OBSERVATIONS ON AN ANTISTAPHYLOCOCCAL MOUSE PROTECTIVE ANTIBODY IN HUMAN SERA

by STEPHEN FISHER

(From the Kanematsu Memorial Institute, Sydney Hospital, Sydney).

(*Accepted for publication 30th August, 1961.*)

SUMMARY.

Significant amounts of antibody capable of protecting mice against lethal intraperitoneal challenge with living staphylococci of strain 90B (Smith), suspended in mucin, were found in 53 out of 100 human sera. Fifty of these sera had been obtained from normal blood donors and 50 from patients suffering from boils; the sera from these two groups contained similar amounts of antibody.

Strain 90B, known in the United States as strain Smith, and three other strains of staphylococci also obtained from the United States resembled one another in the following characters: the formation of mucoid colonies; the slight production of coagulase; high virulence, particularly in mucin suspension, to mice by the intraperitoneal route; and the presence of a common heat-stable component which was highly immunogenic in mice. These characters were not shared with a number of strains of staphylococci originating from Australia and England, and selected to represent the commoner types of pathogenic staphylococci encountered in human medicine and including three strains isolated from bovine mastitis.

Strain 90B (Smith) and similar strains are thus not typical of the commonly occurring staphylococcus pathogenic to man. General conclusions about the pathogenesis of staphylococcal disease in man and resistance to it, drawn from studies on strains of this type, may therefore not be valid.

INTRODUCTION.

It was reported recently (M. W. Fisher, 1959) that a large proportion of human sera and also human gamma globulin are capable of protecting mice passively against lethal challenge with living staphylococci of a strain of high virulence to mice (strain Smith); the challenging organisms were suspended in mucin and injected by the intraperitoneal route. The data also suggested that the protective antibody was present in larger amounts in the sera of normal subjects than in the sera of patients with boils.

The presence of this antibody in human sera and in human gamma globulin was subsequently confirmed; it was not found in the sera of unimmunized mice, rabbits and guinea pigs nor in a goat. Supernatant fluids of the strain used for challenge contained a heat-stable antigen, very small amounts of which were capable of immunizing mice against homologous challenge (S. Fisher, 1960a and b).

It thus seemed possible that antibodies to the heat-stable antigen might be of significance in resistance to staphylococcal disease in man. The work described below was undertaken (1) to examine a further series of human sera for the presence of antibody capable of protecting mice against a challenge by strain 90 (Smith), (2) to examine representative strains of staphylococci for the presence of a heat-stable immunogenic antigen active in high dilution, characteristic of strain 90B (Smith). In other words, is it justifiable to draw any general conclusions about staphylococcal immunity from experiments with strain 90B (Smith)?

Materials and Methods.

Strains of staphylococci.

Unless otherwise stated, all were coagulase positive.

Strain 2299: Cowan Type I, obtained from Dr. J. Brodie, City Hospital, Aberdeen, Scotland.

Strain N.C.T.C. 8531: Cowan Type II type strain.

Strain N.C.T.C. 8532: Cowan Type III type strain.

Strain N.C.T.C. 8178 ("Newman"), used in coagulase studies (Duthie, 1952 and 1954; Duthie and Lorenz, 1952).

Strain Wood 46: a well-known strain used for alpha haemolysin production.

Strain 1531: isolated from meninges in Melbourne in 1957.

3 strains P.S.80, 7530 and 8248: phage type 80/81 strains supplied by Dr. Phyllis Rountree, Royal Prince Alfred Hospital, Sydney.

3 strains K4023-5, K3551-25 and L125-2: isolated from bovine mastitis, supplied by Mr. G. C. Simmons, Animal Research Institute, Yeerongpilly, Brisbane.

12 strains F1 to F12: twelve unselected strains isolated recently from outpatients at Sydney Hospital, Sydney.

4 strains from the United States: two (strains 90B and S193N4) were kindly sent by Dr. M. W. Fisher, of Parke Davis and Co., Detroit. The history of strain 90B (Smith) has been described by the author in earlier reports (Fisher, 1960a and b). Two strains, U.C.76 and U.C.746 ("Fritchie"), were kindly supplied by Dr. C. W. DeWitt, of the Upjohn Co., Kalamazoo, Michigan. Strain U.C.76 was referred to by DeWitt (1958), and strain U.C.746 by Higginbotham and Bass (1961). The general characters of these strains, as found in this laboratory, are shown in Table 1.

The strains, unless used immediately after isolation (Strains F1 to F12), were maintained on nutrient agar slopes under paraffin oil.

Sera.

Samples of venous blood were obtained from Red Cross blood donors and from Sydney Hospital outpatients suffering from boils.

Gamma globulin prepared from the sera of normal blood donors and containing no preservative was obtained from the Commonwealth Serum Laboratories, Melbourne.

Preparation of staphylococcal suspensions for the inoculation of mice.

The bacteria were grown overnight on nutrient agar slopes and suspended in saline, using Brown's turbidity tubes to adjust the turbidity of the suspension. To prepare suspensions in clotting mixture (Boake, 1956; Fisher, 1957), saline suspensions were mixed with equal volumes of a solution containing 2 p.c. human fibrinogen and 2 p.c. fresh human plasma

TABLE 1.

Characters of American strains

Strain	Colonies on human blood agar			Production of coagulase		Fermentation of carbohydrates							Bacteriophage type
	Colour	Whether mucoid	Haemolysis	Slide test	Tube test	Glucose	Maltose	Mannite	Dulcitol	Salicin	Sucrose	Lactose	
90B (Smith)	Mod. golden	Moderately	Moderate	—	+	A	A	A	—	—	A	A	Untypable
S193 N4	Buff	Slightly	Slight	—	+	A	A	A	—	—	A	A	71
U.C. 76	Mod. golden	Moderately	Moderate	—	—	A	A	A	—	—	A	A	71
U.C. 746	Buff	Moderately	Moderate	—	+	A	A	A	—	—	A	—	3B, 71

in saline; the mixture was made immediately before inoculation. For the preparation of suspensions in mucin, one volume of bacterial suspension in saline was mixed with ten volumes of 5 p.c. hog gastric mucin (Type 1701W, the Wilson Laboratories, Chicago), made up according to the manufacturer's instructions.

Preparation of suspension of heated staphylococci for the absorption of gamma globulin.

Staphylococci were grown overnight on nutrient agar in flat 20 oz. bottles. The condensed liquor was discarded and the growth washed off with 3 ml. saline per bottle. The suspension was filtered through cotton wool, centrifuged at 10,000 r.p.m. for 30 minutes at 5° C. in tubes of known weight and the supernatant fluid discarded. After determining the wet weight of the organisms, 100 mg. per ml. were suspended in sterile saline. The suspensions were placed in a boiling water bath for 10 minutes, allowed to cool and diluted, when required, in sterile saline in tenfold steps.

Immunization and challenge of the mice.

For active immunization, 0·5 ml. antigen (culture supernatant fluid of strain 90B) (Fisher, 1960b) was injected subcutaneously over the back and the mice challenged 10 days later. For passive immunization, 0·2 ml. serum dilution or 0·4 ml. gamma globulin dilution was injected, and the mice challenged next day.

For challenge, 1 ml. bacterial suspension was injected intraperitoneally. The animals were observed for seven days after inoculation.

In all experiments, adult white mice of either sex were used.

RESULTS.

Virulence of 16 *strains of staphylococci to mice* (Table 2).

The first twelve strains listed were of Australian or English origin; the last four were strains obtained from America.

Each strain was tested in a separate experiment and each was suspended in saline, clotting mixture and mucin. In saline and clotting mixture the number of bacteria injected ranged from 5×10^9 to 5×10^7, and in mucin suspension from 5×10^8 to 5×10^2.

Every one of the first twelve strains was more virulent in clotting mixture than in saline and the difference for the whole group was statistically significant ($0{\cdot}01 < p < 0{\cdot}02$). There was no difference between the corresponding groups inoculated with the last four strains: the survival rates in both these groups were lower than in the mice inoculated with the first twelve strains suspended in clotting mixture. Mucin enhanced the virulence of all strains; this effect was significantly greater on the last four (the American) strains ($p < 0{\cdot}01$).

Characters of staphylococci of American origin.

In view of the unusual virulence of the American strains their general characters were investigated (Table 1). They were found to be somewhat mucoid and appeared to produce only free coagulase or none. Three out of four were lysed by phages of the standard typing set used for human pathogens. Their characters warrant the classification of these staphylococci as possible human pathogens.

TABLE 2.

Virulence tests

Strain	Suspending agent and dose (number of organisms)												
	Saline			Clotting mixture			Mucin						
	5×10^{9}	5×10^{8}	5×10^{7}	5×10^{9}	5×10^{8}	5×10^{7}	5×10^{8}	5×10^{7}	5×10^{6}	5×10^{5}	5×10^{4}	5×10^{3}	5×10^{2}
	Number of mice surviving in each group of 5												
2299	3	5	5	0	1	5	0	0	3	4	4	5	4
N.C.T.C. 8531	1	5	5	0	4	3	0	0	4	5	4	5	5
N.C.T.C. 8532	1	5	3	0	1	3	0	0	0	4	5	4	5
N.C.T.C. 8178	5	5	5	0	5	5	0	0	5	5	4	4	4
Wood 46	0	5	5	0	2	3	0	2	5	5	5	5	5
1531	2	5	5	0	0	1	0	2	4	5	5	5	5
PS 80	1	4	5	0	0	5	0	1	5	4	5	5	5
7530	3	5	5	0	0	5	0	0	3	5	4	5	5
8248	0	3	5	0	0	4	0	0	3	4	5	5	5
K4023-5	0	5	5	0	0	1	0	0	4	5	5	4	5
K3551-25	0	4	5	0	0	4	0	0	0	5	5	4	4
L125-2	0	2	5	0	0	0	0	0	0	2	5	4	5
Proportion of survivors	127/180 (71%)			52/180 (29%)			262/420 (62%)						
90B	0	0	2	0	0	3	0	0	0	0	0	0	0
S193 N4	0	0	2	0	0	3	0	0	0	0	0	1	—
U.C. 76	0	1	4	0	0	5	0	0	0	0	0	0	0
U.C. 746	0	0	2	0	0	2	0	0	0	0	0	0	0
Proportion of survivors	11/60 (18%)			13/60 (22%)			1/135 (0·7%)						

Active immunity induced in mice with culture supernatant fluid of American strain 90B (Smith).

Four groups of 10 mice each were immunized with culture supernatant of strain 90B (Smith) grown in sloppy agar in an atmosphere of 80 p.c. oxygen and 20 p.c. CO_2 as described previously (Fisher, 1960b); 0·5 ml. of a 1 in 100 dilution of this antigen was injected. Ten days later one group of these immunized mice and one group of unimmunized mice were challenged with 10^4 organisms suspended in mucin of each of the four American strains.

The results are shown in Table 3. It will be seen that not only is there immunity against strain 90B, but there is cross-immunity of the same order against the other three American strains.

On the other hand, attempts to immunize mice against challenge with strain 90B suspended in mucin, using supernatant of cultures of strain Wood 46 were unsuccessful. In other experiments, however, mice injected with supernatant of strain Wood 46 were immune to challenge with suspensions in clotting mixture of strains 1531 and Wood 46 (Fisher, 1957, 1961 and unpublished work). The supernatants of strain Wood 46 were not active in as high dilutions as the supernatants of strain 90B.

TABLE 3.

Active immunization of mice.

The immunization consisted of one subcutaneous injection of 0.5 ml. of strain 90B supernatant fluid diluted 1/100 in saline.

Challenge strain	Survivors/total	
	Immunized mice	Unimmunized controls
90B	10/10	1/10
S193 N4	9/10	0/10
U.C. 76	10/10	1/10
U.C. 746	10/10	1/10

Passive immunity to challenge with strain 90B in mucin conferred on mice by 100 human sera.

Fifty sera from normal blood donors and 50 sera from patients suffering from boils were tested for their content of mouse protective antibody against challenge with 10^4 organisms of strain 90B suspended in mucin (100 LD50's). 0·2 ml. of serum diluted 1 in 10 was injected and the mice challenged the next day. Five mice were used for each serum; in each experiment, a number of sera from donors and the same number from boil patients were tested. The results are shown in Table 4.

TABLE 4.

Passive immunization of mice with the sera of 50 normal blood donors and of 50 patients suffering from boils (8 experiments).

	Proportion of surviving mice per serum	Number of sera	Total proportion of survivors	Mean number of mice surviving out of 5 per serum
Sera from blood donors	0/5	16		
	1/5	8		
	2/5	3		
	3/5	4	114/250	2·28
	4/5	7		
	5/5	12		
	Total	50		
Sera from boil patients	0/5	14		
	1/5	9		
	2/5	8	101/249	2·06
	3/5	5		
	4/5	5		
	5/5	9		
	Total	50		
Unimmunized controls			4/80	

In the dilution used, both groups of sera protected the mice significantly ($p < 0{\cdot}01$); there was no difference between the protection given by the serum of normal individuals and that given by the serum of patients suffering from boils.

Absorption of protective antibody from gamma globulin by various strains of staphylococci.

It was known from previous work (Fisher, 1960a) that a certain batch of gamma globulin prepared from pooled normal human serum protected mice against challenge with 100 LD50's of strain 90B suspended in mucin, if 0·2 ml. of 1 in 10 dilution was injected on the day before the challenge.

Boiled suspensions of 28 strains of staphylococci were mixed with an equal volume of 1 in 10 gamma globulin of the batch referred to, refrigerated overnight, and the bacteria sedimented by centrifugation (10,000 r.p.m. for 30 minutes at 5° C.). In the first instance 100 mg. per ml. of bacteria were used for absorption. If there was evidence of absorption of the protective antibody with this dose of bacteria, further absorptions were carried out with tenfold dilutions of the bacterial suspension. Mice were then injected with 0·4 ml. of the gamma globulin dilution which had been exposed to the various strains of staphylococci; next day the mice were challenged as described above.

The results are shown in Table 5. It will be seen that only staphylococci of the four strains of American origin had any capacity of absorbing the protective antibody in gamma globulin against challenge with strain 90B (Smith).

TABLE 5.

Passive protection tests in mice: effect of absorbing diluted gamma globulin with boiled staphylococci of 28 strains (4 experiments).

Absorbing strain	Wet weight of boiled bacteria used for absorption, mg./ml.	Number of mice surviving challenge (for each strain)
2299, N.C.T.C. 8531, N.C.T.C. 8532, N.C.T.C. 8178, Wood 46, 1531, PS 80, 7530, 8248, K4023-5, K3551-25, L125-2, F1, F2, F3, F4, F6, F7, F8, F9, F11	100	5/5
F5, F10, F12	100	4/5
90B	100	0/5
	10	0/5
	1	1/10
	0·1	10/10
S 193 N4	100	0/5
	10	0/5
	1	0/5
	0·1	0/5
U.C. 76	100	0/5
	10	0/5
	1	0/5
	0·1	0/5
U.C. 746	100	0/5
	10	0/5
	1	0/5
	0·1	0/5
Unabsorbed gamma globulin		19/20
Controls (no gamma globulin given)		1/40

DISCUSSION.

The studies described above and those reported earlier (Fisher, 1960b) have shown that the four American strains of staphylococci, while classifiable as human pathogens because of their biochemical properties and susceptibility to lysis by certain bacteriophages, differed from the types of pathogenic staphylococcus commonly isolated from human patients in producing mucoid colonies and in possessing a heat-stable antigen not shared with ordinary strains. This antigen appeared to confer on these organisms exceptionally high virulence to mice when injected intraperitoneally, particularly if the cells were suspended in mucin. The antigen was present in culture supernatant fluids, very small amounts of which were capable of immunizing mice against lethal intraperitoneal challenge with mucin suspensions of the homologous organism; it was also demonstrable in boiled bacterial suspensions which could be used to absorb the mouse protective antibody from sera.

In passive protective tests on 100 human sera 43 p.c. of the immunized animals survived, as against 5 p.c. of the unimmunized mice. This finding confirms the wide distribution in human sera of the mouse protective antibody against mucin-enhanced challenge with strain Smith (90B) (M. W. Fisher, 1959). However, in this series, sera from normal blood donors and sera from boil patients contained similar amounts of antibody, although the amount of serum used for immunization was chosen to permit the detection of small differences in protective antibody content. This antibody thus appears to be of little significance in the prevention of boils; its rôle in the prevention of other forms of staphylococcal disease has not been investigated.

Antibodies to other types of staphylococci may be as widely distributed in human sera as the mouse protective antibody against strain Smith (90B), but their demonstration may be impossible by mouse protection tests, owing to the large numbers of organisms of strains of the usual types necessary for challenge, even in the presence of mucin. Nevertheless, strains of apparently ordinary virulence have been used in mucin suspension for challenging mice immunized actively with heat-killed staphylococci (Stamp, 1953, 1956).

It is clear that the American strains investigated represent an uncommon, perhaps unique, group of staphylococci. It would seem unwise to draw generalised conclusions about the pathogenesis of staphylococcal infections from studies of strains belonging to this group, interesting though these organisms are.

Acknowledgments. I am indebted to Dr. Phyllis Rountree for the bacteriophage typing, to Dr. R. J. Walsh for samples of blood and to Dr. H. K. Ward for the gamma globulin. Technical assistance was provided for under a research grant from the Postgraduate Medical Foundation, University of Sydney.

REFERENCES.

Boake, W. C. (1956): J. Immunol., 76, p. 89.
DeWitt, C. W. (1958): J. Bact., 76, p. 631.
Duthie, E. S. (1952): J. gen. Microbiol., 7, p. 320.
Duthie, E. S. (1954): *Ibid.*, 10, p. 427.
Duthie, E. S., and Lorenz, L. L. (1952): *Ibid.*, 61, p. 95.
Fisher, M. W. (1959): Nature, 183, p. 1692.
Fisher, S. (1957): *Ibid.*, 180, p. 1479.
Fisher, S. (1960a): Austral. J. exp. Biol., 38, p. 339.
Fisher, S. (1960b): *Ibid.*, 38, p. 479.
Fisher, S. (1961): Med. J. Austral., in press.
Higginbotham, R. D., and Bass, J. A. (1961): Texas Rep. Biol. Med., 19, p. 283.
Stamp, Lord (1953): Lectures on the Scientific Basis of Medicine, 3, p. 279.
Stamp, Lord (1956): Personal communication.

THE EXCRETION OF β-AMINOISOBUTYRIC ACID FOLLOWING THYMINE AND DIHYDROTHYMINE ADMINISTRATION TO MAN

by B. L. HILLCOAT

(From the Department of Pathology, Brisbane Hospital, Brisbane).

(*Accepted for publication 16th August, 1961.*)

SUMMARY.

A study of the excretion of β-aminoisobutyric acid (BAIB) after oral administration of thymine and of dihydrothymine (DHT) to a series of human subjects including normal, splenectomised and leukemic individuals, suggests that the spleen may influence this excretion. The leukemic patients studied differ from normal in their lower excretion of BAIB after DHT ingestion.

INTRODUCTION.

β-Aminoisobutyric acid (BAIB) has been shown by Fink and others (1952, 1954, 1956) to be a metabolic breakdown product of thymine, through dihydrothymine (DHT) and β-ureidoisobutyric acid. The amino acid may be excreted in the urine and its appearance in large amounts therein has been reported in various conditions. Gartler (1959), by feeding various pyrimidine precursors, has demonstrated that only thymine and dihydrothymine are significant precursors of urinary BAIB in man. Hillcoat (1960a) reported the excretion of large amounts of BAIB in the urine of three patients with thalassemia major, but a much lower excretion in two patients with the same disease whose spleens had been removed. Consequently an investigation has been begun in an attempt to determine whether the spleen is related to the excretion of this amino acid.

Materials and Methods.

Commercial samples of thymine and DHT obtained from the Sigma Chemical Company were administered in capsules containing 500 mg. for adults and 250 mg. for children. The capsules were taken before breakfast, after a fasting urine specimen had been collected, and urine samples were collected after 2, 4, 6 and 8 hours. The BAIB was separated chromatographically and its concentration recorded in units as previously described (Hillcoat, 1960b).

Five normal adults (Cases 1, 2, 3, 4 and 5) and three normal children (Cases 6, 7 and 8) ingested DHT; three normal adults (Cases 1, 2 and 3) thymine; four patients (Cases 13, 14, 15 and 16) whose spleens had been removed for traumatic rupture and three (Cases 17, 18 and 19) whose spleens had been removed for thalassemia major, DHT; three leukemic patients (Cases 9, 10 and 11), both DHT and thymine and one (Case 12) DHT alone. The cases of thalassemia major were those reported previously (Hillcoat, 1960a).

The case histories of the leukemic patients studied were as follows:

Case 9. A 53-year-old male patient was diagnosed as a case of monocytic leukemia with a hypercellular marrow and active erythropoiesis. He did not respond to splenic radiation to his moderately enlarged spleen.

Case 10. A 2½-year-old girl with lymphoblastic leukemia had two remissions on drug therapy. Her spleen was moderately enlarged.

Case 11. A 71-year-old man with monocytic leukemia showed a high white cell count in the peripheral blood, a large spleen and "hypersplenism" evidenced by a falling hemoglobin level with hyperplasia of red cell precursors in the marrow, and nucleated red cells in the peripheral blood. On treatment with myleran he responded well. No BAIB was present in his urine.

Case 12. A 12-year-old boy was diagnosed as suffering from acute lymphoblastic leukemia with a low white cell count in his peripheral blood and moderate splenomegaly. He showed some response to transfusion and treatment with TEM.

RESULTS.

The results are shown in Tables 1 and 2. From Table 1 it can be seen that in both normal adults and children thymine and DHT each produce a peak of BAIB excretion (3, 4 or 5 units) 2 to 4 hours after administration. Case 8 is an exception to this and shows a low excretion of 2 units. Table 2 shows that in leukemic cases 9, 10 and 11 the excretion of BAIB following DHT administration is low whereas following thymine administration it is normal. Case 12 showed a normal excretion of BAIB after DHT. Cases 13 to 19, the splenectomised series, show a lowered excretion of BAIB except in Case 14 where a peak of 3 units at 2 hours occurs.

TABLE 1.

Normal subjects.

Time of urine collection after ingestion of compound	Case number										
	1		2		3		4	5	6	7	8
	*THY	†DHT	THY	DHT	THY	DHT	DHT	DHT	DHT	DHT	DHT
Pre-test	0	0	0	0	0	0	0	0	0	0	0
2 hours	3	3	3	0	4	2	0	4	3	1	trace
4 hours	5	4	5	4	2	4	4	3	2	3	2
6 hours	2	1	3	3	1	2	4	1	1	1	trace
8 hours	2	0	2	1	0	1	2	0	0	0	0

* THY = Thymine.
† DHT = Dihydrothymine.
0 = none detected

TABLE 2.

Leukemic and splenectomised subjects.

Time of urine collection after ingestion of compound	Case number of leukemic subjects							Case number of splenectomised subjects						
	9		10		11		12	13	14	15	16	17	18	19
	*THY	†DHT	THY	DHT	THY	DHT	DHT	DHT	DHT	DHT	DHT	DHT	DHT	DHT
Pre-test	0	0	0	2	0	trace	0	0	0	0	0	2	1	1(0)
2 hours	3	2	4	2	3	1	3	2	3	0	0	2(0)	2(1)	2(1)
4 hours	2	1	3	1	3	1	4	1	2	trace	trace	2(0)	2(1)	—
6 hours	0	0	1	2	0	1	2	trace	trace	2	trace	2(0)	3(2)	1(0)
8 hours	0	0	0	trace	0	1	0	0	0	0	0	2(0)	2(1)	—

* THY = Thymine.
† DHT = Dihydrothymine.
( ) = net excretion = test excretion minus pre-test excretion.
0 = none detected.
— = not examined.

DISCUSSION.

The absorption and metabolism of DHT and thymine, and the subsequent excretion of BAIB in the urine, involve so many variables that the significance of departures from the normal is difficult to assess. DHT has an optically active carbon atom at position 5 and hence the commercially available preparation would be in the DL-form. It is unknown how the body handles these isomers in absorption and metabolism. The BAIB isolated from the urine of human subjects by Crumpler, Dent, Harris and Westall (1951), Fink (1951), and Fink, Henderson and Fink (1951), was laevorotatory as was that found in iris bulbs by Asen, Thompson, Morris and Irreverre (1959). Whether D-BAIB results from D-DHT, and how the kidney would handle D-BAIB, is not known. Gartler (1959) and Fink (1956) do not consider this point although they use synthetic DHT in enzymatic investigations and draw conclusions from quantitative data. According to Fink's findings of the degradation of thymine to dihydrothymine to β-ureidoisobutyric acid to β-aminoisobutyric acid in the rat, it might be expected that DHT would be a better precursor of BAIB than thymine but our results show that this is not the case. This may be explained by a species difference, or by the fact that thymine has no optically active carbon atom, whereas the DHT consists of two isomeric forms, one of which may not be metabolised as well as the other.

The failure of splenectomised patients to excrete as much BAIB after DHT ingestion as normal individuals suggests that the spleen may play a rôle in the process. This supports the observation (Hillcoat, 1961) that splenectomy reduced the excretion of BAIB in a patient with thalassemia major. What this rôle would be is purely speculative. An effect on intestinal or cellular absorption or renal excretion appears unlikely, but several facts suggest the spleen may be associated with nucleic acid metabolism or catabolism. The depressant effect on bone marrow activity of a large spleen and the "release" of nucleated red cells into the circulation following splenectomy is well known. A deoxyribonuclease has been described in the spleen (Koerner and Sinsheimer, 1957) and the spleen is known to sequestrate nucleated red cells (Crosby, 1959) and can remove the nuclear material from red cells which are left intact (Vorhaus and Turner, 1957). Also the organ is ideally situated anatomically for "pretreating", as it were, substances which could then be carried via the portal vein to the liver.

In leukemia, the results suggest a normal degradation of thymine with a less effective catabolism of DHT. This may not be related to the spleen, but rather to a disturbance of pyrimidine metabolism in this condition, as, for example, a more ready uptake of DHT into nucleic acids in leukemic patients than in the normal.

REFERENCES.

Asen, S., Thompson, J. F., Morris, C. J., and Irreverre, F. (1959): J. biol. Chem., 234, p. 343.
Crosby, W. H. (1959): Blood, 14, p. 399.
Crumpler, H. R., Dent, C. E., Harris, H., and Westall, R. G. (1951): Nature, 167, p. 307.
Fink, K. (1951): Proc. Soc. exp. Biol. (N.Y.), 76, p. 692.
Fink, K. (1956): J. biol. Chem., 218, p. 9.
Fink, K., Henderson, R. B., and Fink, R. M. (1951): *Ibid.*, 78, p. 135.
Fink, K., Henderson, R. B., and Fink, R. M. (1952): J. biol. Chem., 197, p. 441.
Fink, K., and McGaughey, C. (1954): Fed. Proc., 13, p. 207.
Gartler, S. M. (1959): Arch. Biochem. Biophys., 80, p. 400.
Hillcoat, B. L. (1960a): Austral. J. exp. Biol., 38, p. 441.
Hillcoat, B. L. (1960b): Austral. Ann. Med., 9, p. 276.
Hillcoat, B. L. (1961): In press.
Koerner, J. F., and Sinsheimer, R. L. (1957): J. biol. Chem., 228, p. 1039.
Vorhaus, E. F., and Turner, J. C. (1957): Blood 12, p. 469.

CHANGES IN CARDIAC CONTRACTILITY EVOKED UNDER ISOTONIC AND ISOMETRIC CONDITIONS[1]

by WINIFRED G. NAYLER

(From the Baker Medical Research Institute, Melbourne).

(*Accepted for publication 3rd August, 1961.*)

SUMMARY.

Isolated ventricles or ventricular strips from winter toads (*Bufo marinus*) were used to study changes in contractile behaviour of cardiac muscle under isometric and isotonic conditions. Variations in the stimulation rate and in the Na^+ and Ca^{++} concentrations of the perfusate were used to induce changes in contractility.

The changes recorded isotonically differed from those recorded isometrically. The optimum stimulation frequency was different in the two preparations but the rate at which the various responses were produced depended to some extent upon the stimulation rate used. The results are discussed in relation to the hypothesis that stimulation and stretch influence calcium exchangeability.

INTRODUCTION.

Henrotte, Cosmos and Fenn (1960) when determining the rates of calcium exchange in stretched and non-stretched turtle (*Pseudemys elegans*) ventricles noted that stretching enhanced the outward calcium exchange. In the same study these workers observed that whereas stretching retarded the onset of the hypodynamic state and suppressed or abolished the staircase these phenomena were readily evoked in non-stretched ventricles, an observation which led them to suggest that stretching suppressed the staircase by increasing the quantity of calcium exchanged and therefore available for participation in the excitation-contraction mechanism.

Previously Niedergerke (1957) postulated that calcium facilitation of cardiac contractility involved a surface-acting mechanism, an hypothesis which was extended by Lüttgau and Niedergerke (1958) to include the calcium-sodium antagonism originally described by Daly and Clark (1921). If, as the results of Henrotte *et al.* indicate, the accumulation at or loss from the superficial layers of the heart of these ions is influenced by the degree of stretch applied then phenomena which depend upon such surface binding activities should vary in magnitude according to the use of freely contracting isotonic hearts or of isometric strips to which a constant resting tension is applied.

[1] The expenses of this investigation were defrayed by a grant from the Life Insurance Medical Research Fund of Australia.

The experiments described below were carried out in an attempt to determine whether or not the changes in contractility which are believed to result from changes in the surface concentrations of calcium ions, namely, the positive inotropic effect of calcium, the effect of sodium depleted Ringer solution and the classical staircase associated with changed stimulation frequencies, vary according to the use of freely contracting isotonic or constant length isometric preparations. Isolated ventricles or ventricular strips from winter toads (*Bufo marinus*) were used.

Method.

The isolated ventricles and ventricular strips used in these studies were removed from unselected toads between May and September. Experiments were performed at room temperature (19-22° C.) and both isotonic and isometric preparations were stimulated directly with supramaximal rectangular pulses of 1 millisec. duration, delivered from a Tektronix square wave generator. Unless otherwise stated a modified Ringer solution of the following composition was used:

	mM.
NaCl	115
Na HCO_3	20·6
Na $H_2PO_4.2H_2O$	3·0
$MgSO_4$ $7H_2O$	2·2
Glucose	16·5
KCl	3·2
$CaCl_2$	1·3

The pH of this medium, through which a 95 p.c. O_2-5 p.c. CO_2 mixture was continuously bubbled, was 7·4.

An equilibration period of thirty minutes following dissection was allowed during which time both isometric and isotonic preparations were stimulated at a rate of 6 beats per minute. In some additional isotonic experiments a stimulation rate of 30 beats per minute was used throughout the equilibration period to ensure that the heart was hypodynamic.

The photo-electric apparatus previously described (Nayler and McCulloch, 1960) was used to record the isotonic contractions of the stimulated isolated ventricles. In essence this consists of a thin rigid wire introduced into a cannula and placed in the aorta so that the beaded end of the wire lies against the endocardium. At the free end of the wire a very light shutter lies directly in the path between a constant light source and a selenium barrier layer photo-electric cell (1 cm. × 1·5 cm.). Displacement of this shutter during the successive contractions and relaxations varies the light falling on the photo-electric cell, the output from which is amplified by means of a linear D.C. amplifier (gain 0-300) and recorded on a direct inking Evershed and Vignoles recorder. Over the frequency range used in this study the response of this recorder was linear.

The cannula, constructed of perspex, was designed so that the fine thin-walled section which was inserted into the aorta communicated directly with an overhead wider bore reservoir. Care was taken during the construction of the cannulae to ensure that the internal vertical walls were parallel and in the setting-up of each particular experiment any contact between the rigid steel wire carrying the shutter and the cannula walls was avoided. The shutter was cut from very thin light-weight metal sheet to avoid unnecessary inertia. The introduction of the cannula tip into the lumen of the aorta necessarily destroys the aortic valves so that the diastolic volume and hence the resting fibre length is governed by the

extent of filling. Using the diastolic base line recorded at the conclusion of the equilibration period as the index of ventricular volume, the height of the fluid in the cannula reservoir was adjusted for each particular set of experiments so that a constant diastolic volume and hence resting fibre length was maintained throughout the duration of any experiment. When high rates of stimulation were used (60 beats/minute) it was not possible to maintain the diastolic volume at that level which was previously recorded in the same preparation stimulated at the basal rate (6 beats per minute), presumably because the time interval which separated successive relaxations and contractions was too short to permit complete relaxation. This condition was recorded as an ascending diastolic baseline and was readily apparent (see staircase results).

Tensions produced during isometric contractions of stimulated ventricular strips (approx. 1·5 mm. in thickness and 20 mm. in length) were recorded with an R.C.A. 5734 mechano-electrical transducer as previously described (Nayler, 1960a). This apparatus was essentially the same as that described by Abbott and Mommaerts (1959), the strips being maintained under a constant resting tension throughout the duration of the experiment. A preliminary study indicated that if the initial tension applied was less than 5 gm. (calibrated by suspending this weight from the side arm attached to the transducer) then the muscle strip showed visible movement during contraction and was therefore no longer isometric. When a resting tension equivalent to the 5 gm. weight was applied the strip remained fully active under isometric conditions for many hours. Consequently, unless otherwise stated, a resting tension equivalent to 5 gm. weight suspended from the side arm was applied and maintained throughout each experiment. Tension-duration curves were displayed on a 5-in. cathode ray oscilloscope and recorded either photographically or directly on an Evershed and Vignoles recorder. The whole apparatus was checked for linearity and stability.

The "staircase".

A series of experiments were carried out in which the staircase effect was evoked in isometric and isotonic preparations by stimulation at a variety of frequencies ranging from 0·5 to 60 beats/minute. Both preparations were initially stimulated for 30 minutes at the basal rate of 6 beats/minute. The twitch height of the isotonic and the twitch tension of the isometric series were recorded after each preparation had been stimulated for ten minutes at a frequency within the above range and that frequency which gave the maximum activity noted for both studies. In some other isotonic experiments the effect on the staircase of preliminary stimulation at the rate of 30 beats/minute throughout the half hour equilibration period was determined.

In additional experiments performed under isometric conditions the concentration of Ca^{++} ions in the Ringer solution was reduced to 0·425 mM. and the frequency-tension response compared with that recorded in the same preparations at the same resting tension when Ringer containing 1·3 mM. Ca^{++} was used.

Na^+ depletion.

Stimulated isometric (resting tension 5 gm.) and isotonic preparations were initially perfused for 30 minutes with Ringer solution containing 0·425 mM. Ca^{++}. After equilibration the perfusate was replaced by another which still contained 0·425 mM. Ca^{++} but whose Na^+ concentration had been reduced by half to 57·5 mM., tonicity being maintained with Li^+ as lithium chloride. The resultant positive inotropic response was recorded and after the new equilibrium had been established (at the same resting tension or diastolic volume) the preparation was re-perfused with Ringer solution the Ca^{++} concentration of which was 0·425 mM. and the Na^+ concentration 115 mM. and the experiment then repeated at another stimulation rate. The response to this Na^+ depletion was usually determined at four separate stimulation frequencies in any one preparation and the response calcu-

lated as the percentage increase, relative to the amplitude of contraction or the tension previously recorded in that preparation perfused with Ringer solution whose Ca^{++} and Na^{+} concentrations were 0·425 mM. and 115 mM. respectively and which was stimulated at the same rate and at the same resting tension or diastolic volume. The results obtained from the first ionic substitution were discarded and, in an effort to check against changes associated with the progressive onset of the hypodynamic state, the above ionic substitution was repeated at the conclusion of each study at that stimulation frequency which was used in the first recorded substitution. Provided that the partial replacement of Na^{+} by Li^{+} was limited numerically as described above, normal contractile behaviour was recorded when Ringer containing 0·425 mM. Ca^{++} and 115 mM. Na^{+} was re-introduced, i.e. there was no marked loss of contractility such as is recorded following the repeated replacement of Li^{+} for Na^{+} in the perfusate.

Additional experiments were performed to determine the effect of dinitrophenol ($1{\cdot}09 \times 10^{-5}$M) of sodium iodoacetate (5×10^{-5}M) and of the absence of Mg^{++} on the inotropic response recorded in the Na^{+} depletion experiments. In the presence of the metabolic inhibitors isometric preparations were stimulated at a rate of 6 beats per minute and the isotonic ventricles at 30 beats per minute since preliminary experiments had shown that the response to Na^{+} depletion was maximal at these frequencies.

Ca^{++} depletion.

In similar experiments the Ca^{++} concentration of both isotonic and isometric preparations stimulated at 6 beats/minute was reduced from 1·3 mM. to 0·425 mM. and the resultant changes in twitch tension and the amplitude of contraction recorded. The slight osmotic change associated with this Ca^{++} deletion was disregarded. As described above diastolic volumes and resting tensions were kept constant in the isotonic and isometric series respectively.

RESULTS.

The results recorded throughout these experiments showed a quantitative but not a qualitative dependence upon the type of preparation used; isotonic conditions apparently favoured the development of the positive inotropic response associated with a raised Ca^{++} concentration or reduced Na^{+} concentration in the perfusate.

Staircase.

Although the classical staircase was recorded following frequency changes in both the isotonic and isometric preparations the rate of stimulation at which maximum tension was produced under isometric conditions as used in this study was significantly different from that at which the maximum amplitude of contraction was recorded during isotonic contractions. This difference is shown in Fig. 1 where the mean results of twelve isotonic and six isometric experiments are shown. The Ca^{++} concentration was 1·3 mM. in all experiments. The optimum frequency for the isometric strips was 8 beats per minute compared with 30 beats per minute for both the isotonic preparations and in all cases stimulation at rates above or below the respective optimum level resulted in a decreased tension or twitch height. Over the range of frequencies used in

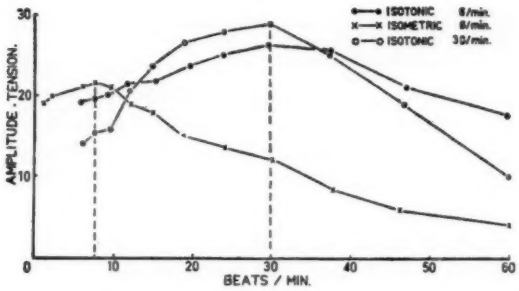

Fig. 1. Mean tensions produced in six isometric preparations and mean amplitudes of contraction developed in twelve other isotonic preparations stimulated at frequencies ranging from 0·5 to 60 beats per minute. All the isometric and six of the isotonic preparations were initially stimulated at the rate of 6 beats/minute during the preliminary equilibration period. The other six isotonic preparations (see key) were stimulated at rate of 30 beats per minute.

these experiments (0·5 to 60 beats per minute) the staircase effect was more pronounced under isotonic than under isometric conditions. As can be seen in Fig. 1, stimulation of the isotonic preparation at the higher rate of stimulation during the initial equilibration period (30 beats per minute instead of 6 per minute) further enhanced the staircase but did not alter the optimum frequency. In both isotonic series constant diastolic volumes could not be maintained throughout the upper limits of the frequency range, the diastolic volume declining when the stimulation rate exceeded 46 beats/minute. Since the amplitude of contraction is determined in part by the resting length of the ventricle some of the decline in contractile amplitudes recorded in those isotonic preparations stimulated at rates exceeding 46 beats/minute may be attributable to this factor. The use of resting tensions in excess of 5 gm. (7, 10 or 12 gm.) for the isometric series depressed the staircase effect but failed to alter the optimum frequency (8 beats per minute).

In those experiments in which the concentration of Ca^{++} in the perfusate was reduced from 1·3 to 0·425 mM. the optimum frequency for the isometric strips changed so that when the Ca^{++} concentration was 0·425 mM. peak tension was produced at a higher stimulation rate than was the case when the Ca^{++} concentration was 1·3 mM. (Table 1). The optimum frequency for the isotonic preparation remained constant whether the Ca^{++} concentration was 1·3 or 0·425 mM. but in both this and the isometric strip the staircase effect was enhanced when the Ca^{++} concentration of the Ringer was reduced.

TABLE 1.

Frequencies at which maximum tensions were recorded in four isometric preparations when [Ca^{++}] was either 1·3 or 0·425 mM.

Prep No.	Optimum frequency	
	[Ca^{++}] = 1·3 mM.	[Ca^{++}] = 0·425 mM.
1.	6/min.	15/min.
2.	6/min.	9·5/min.
3.	3/min.	12/min.
4.	6/min.	12/min.

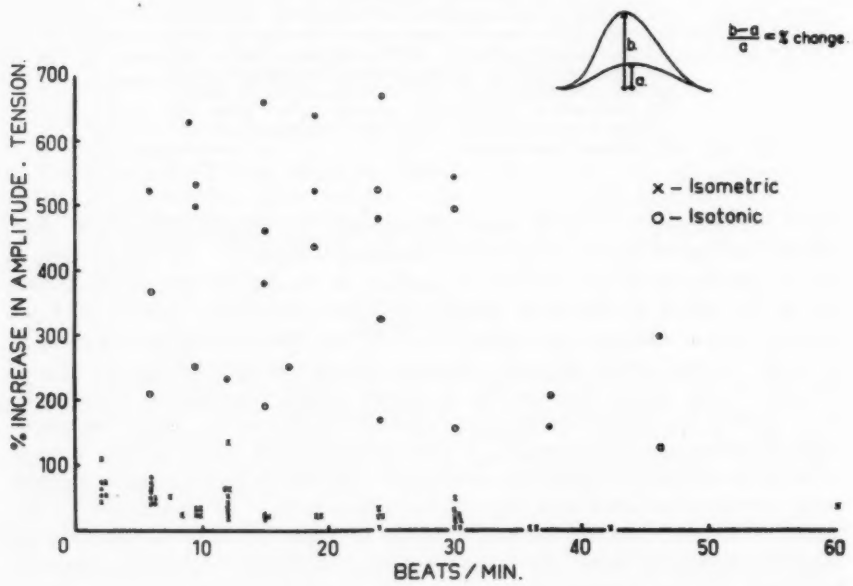

Fig. 2. Changes recorded in tensions and amplitudes of contraction developed in a series of isotonic and isometric preparations following replacement of half the perfusate Na^+ by an equivalent amount of Li^+. The change in the amplitude of contraction and in the tension is expressed as the percentage change relative to the initial tension or contractile amplitude recorded at the particular frequency.

Na^+ depletion.

Replacement of half the perfusate Na^+ by an osmotically equivalent amount of Li^+ resulted in a positive inotropic response, the magnitude of which varied according to whether isotonic or isometric preparations were used.

The overall results of these Na^+ depletion experiments are shown in Fig. 2 where it can be seen that the degree of change recorded in the isometric strips (0-130 p.c.), was consistently less than that recorded under isotonic conditions (130-670 p.c.). The increased amplitude of contraction recorded in the isotonic preparations showed no dependence upon the stimulation frequency used but the change in twitch tension varied directly with the stimulation rate, so making the tension response to Na^+ depletion greater at lower than at higher frequencies. This relationship between the magnitude of the strip's response and the stimulation rate is further displayed in Fig. 3 where the results of Na^+ substitutions carried out on four isometric strips stimulated at a variety of frequencies are summarized.

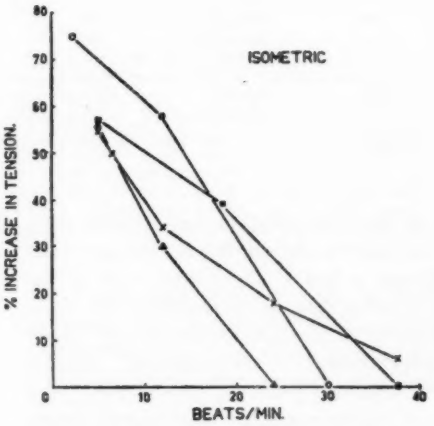

Fig. 3. Percentage increase in tensions recorded in four isometric preparations following replacement of half the perfusate Na^+ by an equivalent amount of Li^+. Substitutions were carried out with each preparation stimulated at several discrete frequencies as indicated by the points on the curves, and the percentage increase in tension calculated as shown in Fig. 2.

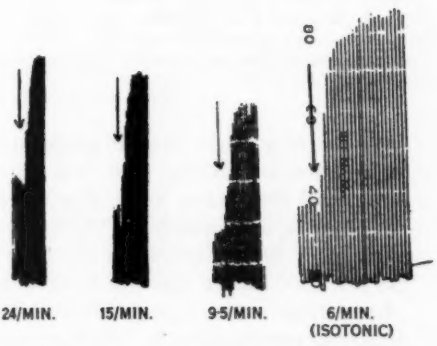

Fig. 4. Increase in the amplitude of contraction of an isotonic preparation following replacement (at arrow) of half the perfusate Na^+ by an equivalent amount of Li^+. The substitution was effected when the preparation was stimulated at 6, 9·5, 15 and 24 beats per minute. Note that the time taken to develop the positive inotropic response varied with the stimulation frequency used. (50 p.c. Na^+-50 p.c. Li^+ Ringer added at arrow.)

In both isotonic and isometric preparations the rate of development of the response to Na^+ depletion was gradual and varied with the stimulation frequency so that when rapid rates were used the response was more quickly developed than was the case at slower stimulation rates. For any one preparation the response appeared to be fully developed throughout a finite number of contractions, irrespective of the stimulation frequency. In Fig. 4, where the

TABLE 2.

Number of beats required to develop the maximum response in Na^+ depletion experiments. Under isometric and isotonic conditions the number of beats required is independent of the stimulation frequency.

Isometric			Isotonic		
No.	Frequency	No. of beats	No.	Frequency	No. of beats
1.	9·5/min. 7·5/min. 6/min.	14 14 14	1.	15/min. 9·5/min. 6/min.	11 11 10
2.	9·5/min. 6/min.	14 14	2.	9·5/min. 6/min.	11 12
3.	9·5/min. 6/min.	24 25	3.	15/min. 9·5/min. 6/min.	16 16 16
4.	7·5/min. 6/min.	20 20	4.	6/min. 30/min.	14 13

response of a single isotonic preparation to Na^+ substitution carried out using stimulation frequencies of 6, 9·5, 15 or 24/min. is shown, it can be seen that the actual time taken to develop the response is less at the higher than at the lower rates of stimulation. At each stimulation rate approximately the same number of beats occurred in the interval between addition of the 50 p.c. Na^+-50 p.c. Li^+ Ringer and the completed development of the response. Similar observations were made in other isometric studies (Table 2). In isotonic and isometric preparations in which stimulation was temporarily interrupted immediately following the addition of Ringer solution in which half the Na^+ had been replaced by Li^+, the positive inotropic response was fully developed only after approximately the same number of contractions had occurred as was previously recorded in the same preparation stimulated continuously. In these experiments stimulation was stopped for periods varying from 10 to 75 seconds. Fig. 5 displays the results of a typical isotonic preparation in which stimulation has been interrupted. In 5A stimulation was stopped for 15 seconds immediately after the 50 p.c. Na^+-50 p.c. Li^+ Ringer had been added but in 5B stimulation was continuous throughout the experiment. In both cases the same number of beats were apparently required to develop the complete response indicating that equilibration of the muscle with the low Na^+ Ringer took place mainly during stimulation or active contraction. No significant difference was found between the number of contractions required by the isometric or isotonic preparations to develop the response to Na^+ depletion, but marked variation did occur between individual preparations.

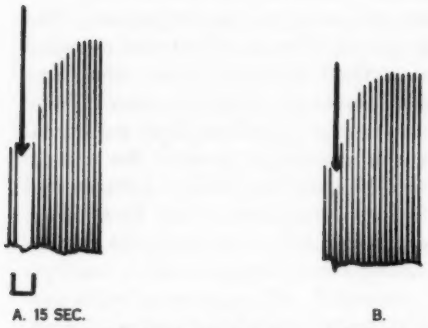

Fig. 5. Positive inotropic response following replacement (at arrow) of 50 p.c. of perfusate Na^+ by Li^+ in an isotonic preparation stimulated at 6 beats per minute. In 5A stimulation was stopped for 15 seconds immediately following the ionic substitution and for a further 10 seconds after the inotropic response had begun. In 5B stimulation was continuous. Note that in 5A and 5B the same number of beats were required for the development of the positive inotropic response.

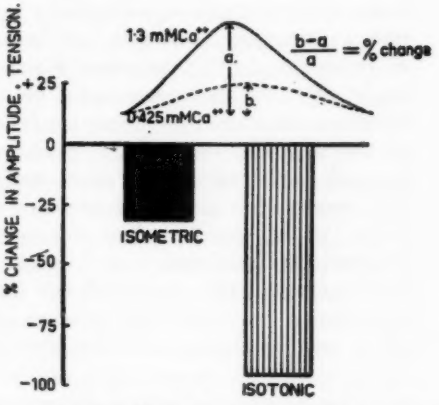

Fig. 6. Negative inotropic response recorded following reduction of Ca^{++} concentration from 1·3 mM. to 0·425 mM. in three isotonic and three isometric preparations. The percentage change was calculated as shown and stimulation rate of 6 beats per minute used in all preparations.

Iodoacetate or the absence of Mg^{++} did not depress the response of either preparation to the reduced Na^+ concentration. In contrast, dinitrophenol, when administered to the preparations 30 minutes or longer prior to the replacement of the perfusate, depressed or abolished the response.

Ca^{++} depletion.

The decreased contractility recorded following the reduction of the Ca^{++} concentration of the perfusate from 1·3 mM. to 0·425 mM. varied in magnitude with the type of preparation used. The results recorded during a series of experiments in which the stimulation rate was 6 beats/minute are summarized in Fig. 6. It is evident that the decline in contractility response due to Ca^{++} depletion was more marked in the isotonic than in the isometric preparations as used in this study.

DISCUSSION.

The results described above indicate that, under the conditions prevailing throughout these experiments, the recorded response of stimulated cardiac tissue contracting isometrically differs quantitatively from that obtained when the contractions are isotonic. In general, isotonic conditions favoured the develop-

ment of the changes in contractility which resulted from the various modifications in the ionic composition of the perfusate and from alterations in the rate of stimulation. This indicates that isotonic rather than isometric conditions might be used advantageously in some physiological investigations. The optimum stimulation frequency for isotonic preparations exceeded that recorded in the isometric series. The phenomena studied included those which are currently explained in terms of the surface accumulation of calcium ions. There is a considerable amount of evidence to favour the hypothesis that some conditions of enhanced cardiac contractility do necessarily involve the surface accumulation of calcium ions at specific sites (Niedergerke, 1957). Lüttgau and Niedergerke (1958) explained the Ca^{++}-Na^{+} antagonism along these lines, arguing that Ca^{++} and Na^{+} compete for a common site on or about the vicinity of the cell membrane. The literature contains other evidence which supports the view that the accumulation of Ca^{++} associated with successive beats provides the basis for the potentiation recorded during the classical staircase (Loewi, 1917; Moulin and Wilbrandt, 1955; Heilbrunn, 1952; Nayler, 1960).

The actual sites at which the proposed binding or accumulation of these ions takes place have not yet been identified but it should be recalled that the earlier micro-incineration studies of Scott (1932) and of Carey and Zeit (1939) revealed the presence of an ash in the A bands, along the surface of the fibres and along the fine transverse lines located in the position of the Z bands. This ash was that of a multivalent ion, presumably calcium and/or magnesium. Recent electron microscopy studies (Porter and Palade, 1957) have demonstrated continuity between the Z band proper and the sarcolemma by way of the endoplasmic reticulum so that there is no morphological reason why changes in the ionic concentrations about the sarcolemma cannot be rapidly relayed to the myofibrils.

The binding of calcium at these sites will be influenced by many factors including the rate at which calcium is released from its bound intracellular sites (Harris, 1957; Hodgkin and Keynes, 1957), the time interval during which the released calcium remains in the vicinity of the binding sites, the area over which the released ions spread and the number of free sites available for calcium uptake. Shanes and Bianchi (1960) have shown that the release of bound intracellular calcium in skeletal muscle may be related to the stimulation frequency. They listed additional evidence which indicated that the action potential could be instrumental in the release of these ions. If it is agreed that the inotropic phenomena investigated in the present study are due to or are associated with the release and subsequent redistribution (surface accumulation) of intracellular calcium ions then the relationship noted between the stimulation frequency and the number of beats required for the complete development of the inotropic responses associated with the various ionic substitutions may be explained in terms of Shanes and Bianchi's results. The apparent absence of any

complication due to "rest contraction" associated with the interrupted stimulation may be due to the reduced concentration of Na^+ ions in the Ringer solution.

The results of Henrotte *et al.* clearly showed that the calcium exchangeability in turtle ventricles was enhanced by stretch. According to their results more calcium will be freely available for participation in the contraction mechanism of stretched than of non-stretched preparations and it follows that any increase in this calcium exchangeability such as may follow an increased rate of stimulation or reduction of the concentration of Na^+ ions in the perfusate will have a greater proportional effect in the non-stretched than in the stretched preparations. Comparison of the present results with those of Henrotte *et al.* indicates that the quantitative differences recorded above between the various isometric and isotonic responses may have been due, in some part at least, to the resting tension applied to the isometric series (the tension applied was the least compatible with the maintenance of isometric conditions and it will be recalled that higher tensions depressed the staircase but did not alter the optimum frequency). Thus, when the calcium exchangeability was already high, as in the stretched isometric strips, the further release of intracellular calcium ions for participation in the contractile mechanism will have a proportionately lesser effect than would be the case if calcium exchangeability was initially low, as in the isotonic non-stretched preparations. The observations of the present study can be explained along these lines.

It is also possible that the active movements of the myofibrils which take place during isotonic, but not during isometric contractions, may contribute to the quantitative differences recorded in the present study between the various responses of the isometric and isotonic series. If, as Ruska, Edwards and Caesar (1958) postulate, the movement of the myofibrils does exert a pump-like effect on the movement of fluid throughout the extracellular and some intracellular spaces (endoplasmic reticulum) then the area over which Ca^{++}/Na^+ exchange can take place will be greater in the isotonic than in the isometric preparations. Such a theory, although speculative, could explain some of the observations made during this present study.

Acknowledgments. I wish to thank Dr. T. E. Lowe for his helpful advice both during these experiments and in the preparation of this paper and Miss J. Howells for her enthusiastic assistance.

REFERENCES.

Abbott, B. C., and Mommaerts, W. F. H. M. (1959): J. gen. Physiol., 42, p. 533.
Bennett, H. S., and Porter, K. R. (1953): J. Anat., 93, p. 61.
Carey, E. J., and Zeit, W. (1939): Proc. Soc. exp. Biol., 41, p. 31.
Daly, I. de Burgh, and Clark, A. J. (1921): J. Physiol., 54, p. 367.
Harris, E. J. (1957): Biochim. et biophysica Acta, 23, p. 80.
Heilbrunn, L. V. (1952): An Outline of general Physiology, Philadelphia, W. B. Saunders Company.
Henrotte, J. G., Cosmos, E., and Fenn, W. O. (1960): Amer. J. Physiol., 199, p. 779.

Hodgkin, A. L., and Keynes, R. D. (1957): J. Physiol., 138, p. 253.
Loewi, O. (1917): Arch. exp. Path. Pharmak., 83, p. 366.
Lüttgau, H. C., and Niedergerke, R. (1958): J. Physiol., 143, p. 486.
Moulin, M., and Wilbrandt, W. (1955): Exper., 11, p. 72.
Nayler, W. G. (1960): J. gen. Physiol., 44, p. 393.
Nayler, W. G., and McCulloch, M. W. (1960): Austral. J. exp. Biol., 38, p. 117.
Niedergerke, R. (1957): J. Physiol., 138, p. 506.
Porter, K., and Palade, G. E. (1957): J. biophys. and biochem. Cytol., 3, p. 269.
Ruska, H., Edwards, G. A., and Caesar, R. (1958): Exper., 14, p. 117.
Scott, G. H. (1932): Proc. Soc. exp. Biol., 29, p. 349.
Shanes, A. M., and Bianchi, C. P. (1960): J. gen. Physiol., 43, p. 481.

THE BEHAVIOUR OF THYMUS GRAFTS IN HIGH AND LOW LEUKAEMIA STRAINS OF MICE[1]

by D. METCALF, N. SPARROW, K. NAKAMURA AND M. ISHIDATE

(Cancer Research Laboratory, Walter and Eliza Hall Institute, Royal Melbourne Hospital, Melbourne).

(*Accepted for publication 29th August, 1961.*)

SUMMARY.

Subcutaneous isografts of whole thymuses in mice of strains AKR and C3H have demonstrated that graft growth is determined partly by the age of the host, but primarily by the age of the graft.

Thymuses from one-week-old C3H mice in six-month-old hosts underwent weight changes synchronous with the growth and involution of the thymus in intact C3H mice.

AKR thymuses grew to a larger size, and exhibited higher mitotic activity than C3H thymus grafts in (AKR × C3H)F_1 hybrid mice.

No changes were observed in the absolute weight or mitotic activity of the lymphoid organs of mice bearing actively growing axillary thymus grafts.

A slight relative weight increase was observed in the axillary lymph nodes draining the graft site, compared with the opposite axillary nodes, but this was not associated with increased mitotic activity in these nodes.

INTRODUCTION.

Interest in the growth of the thymus, and the manner in which lymphopoiesis is regulated in this organ, has been stimulated by the finding that thymectomy prevents the development of lymphoid leukaemia in mice. In the high leukaemia strain AKR, thymectomy greatly reduces the incidence of spontaneous lymphoid leukaemia (McEndy, Boon and Furth, 1944). Regrafting of normal AKR thymus tissue to such mice restores their high incidence of leukaemia (Miller, 1960). In thymectomised (C3H × AKR)F_1 hybrid mice, a high incidence of leukaemia is restored by grafting normal AKR thymic tissue, but not by grafting normal thymic tissue, from mice of the low-leukaemia strain C3H (Law, 1952).

In a comparative survey of AKR and C3H thymuses (Metcalf, 1960), it was found that AKR thymuses grew more rapidly in early life, were heavier, and underwent age involution at a later age, than did thymuses from the low-

[1] This work was supported by the Carden Fellowship Fund of the Anti-Cancer Council of Victoria.

leukaemia strain C3H. Mitotic indices of the primitive lymphoid cells in AKR thymuses were also higher than in C3H thymuses (Nakamura and Metcalf, 1961).

In the present experiments, a study has been made of the growth of normal thymus iso-grafts in AKR and C3H mice, and of AKR and C3H grafts in (AKR × C3H)F_1 hybrid mice and the effects of these grafts on the lymphoid tissues of the host. An attempt has also been made to determine whether the strain differences between AKR and C3H thymuses are due to intrinsic differences in the proliferative activity of these tissues, or are due to differences in circulating regulators of lymphopoiesis in these two strains of mice.

Material and Methods.

Mice.

Mice used were inbred members of the high-leukaemia strain, AKR, and the low-leukaemia strain, C3H. The mice were housed in metal cages with sawdust bedding, in rooms maintained at 75° F. The mice were fed purina chow and water *ad lib.*, with supplemental greens and carrots.

Thymus grafts.

Donor mice were killed with ether, and the thymuses removed aseptically and immersed in saline. Recipient mice were anaesthetised with ether and an incision made through the skin of the dorsal edge of the left axilla. A small pocket in the subcutaneous connective tissues, adjacent to the axillary nodes, was prepared by blunt dissection with forceps. The thymus graft was gently inserted, and the skin wound closed with Michel clips. The delay between thymus removal and grafting never exceeded ten minutes.

Graft and lymphoid organ weights.

The thymus grafts and other lymphoid organs in the recipient mice were dissected free of fat, and weighed on a torsion balance (accuracy ± 0·5 mg.).

Cytology.

Single cell suspensions of thymus grafts were prepared according to the method described by Nakamura and Metcalf (1961). The tissues were minced in glacial acetic acid and stained with orcein and gentian violet. The suspensions of cells were mounted as wet preparations on glass slides. The percentage of large, medium and small lymphocytes in these preparations, and the mitotic indices of the primitive lymphoid cells were determined by counting 5000 consecutive lymphoid cells.

Histology.

Tissues for histological examination were fixed in 10 p.c. Zenker's formalin, dehydrated, blocked in paraffin and sectioned at 7 mμ. Sections were stained with either haematoxylin and eosin, or by the Periodic-Acid Schiff (P.A.S.) technique.

Statistical analysis.

Statistical analyses for significant differences were made using the Student "t" series method.

Standard deviations were calculated according to the formula:

$$SD = \pm \sqrt{\frac{\Sigma \text{ devs. from mean}^2}{n-1}}.$$

RESULTS.

General remarks.

When thymus grafts were examined four or six weeks after implantation, the organ was readily recognisable and had usually maintained its bilobed structure. Commonly, the capsule of the thymus graft was found to be attached to one of the axillary lymph nodes, but histologically, no lymphatic inter-communications appeared to have developed between the two organs.

There appeared to be no particular site of implantation in the axillary region which specifically favoured growth of the graft. Well-developed thymus grafts were not surrounded by a prominent network of blood vessels.

Following grafting, massive death of the cortical lymphoid cells occurred. By day 5 after grafting, the grafts averaged 1-5 mg. in weight, and were com-

Fig. 1. One-week-old C3H thymus four weeks after grafting to a six-month-old male C3H host. Note the presence of islands of thymic tissue within the general outline of the thymus lobe. P.A.S. Stain. X 100.

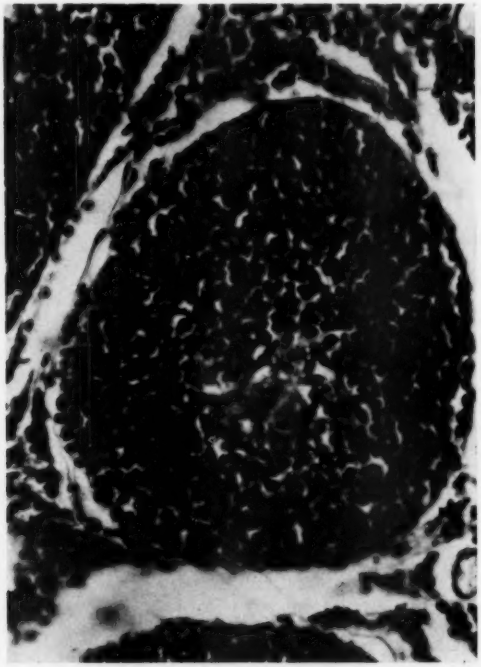

Fig. 2. Higher magnification of same thymus graft, showing epithelial cells at centre of island of thymic tissue. P.A.S. Stain. X 500.

posed of surviving epithelial and reticular elements. After this time, atypical lymphoid cells re-appeared under the capsule, and mitotic activity commenced. By day 10, thymus grafts had developed a structural pattern, typical of thymus tissue. Maximal weight increase of the graft was achieved by the third to fifth week after grafting. These results are in agreement with those reported by Carnes *et al.* (1956).

Although thymus grafts maintained the general bilobed structure of thymus, histologically, the graft was commonly broken up into multiple lymphoid islands. This may have been the result of injury to the organ during transplantation. The insular nature of the thymus grafts was well seen in sections stained with P.A.S. (Fig. 1). At the centre of each island of lymphoid cells, a group of epithelial cells was seen (Fig. 2). Thus these islands replicated, in miniature, the original structure of the thymus lobe.

Effect of age of host on growth of thymus grafts.

The effect of the age of the host on the growth of thymus grafts was investigated using, as donor material, one-week-old thymuses from AKR and C3H mice.

In the first series, one-week-old female AKR thymuses, weighing 18 mg., were grafted subcutaneously to female AKR mice, aged either four weeks, two months or four months. Twenty-five days after grafting, the recipient mice were killed and the weights of the thymus grafts determined. Fig. 3 shows the results obtained. The mean graft weight in the four-week hosts was 34 ± 21 mg., significantly higher than that of grafts in two-month-old hosts (19 ± 17 mg.), and in the four-month hosts (17 ± 17 mg.). These results suggest that thymus grafts are exposed to a greater growth stimulus in young hosts. It should be noted that thymus age involution begins in female AKR mice at eight weeks (Metcalf, 1960). The first group of grafts in four-week hosts therefore represents graft growth in preinvolutional hosts; the other two groups, growth in postinvolutional hosts.

In all groups, a varying percentage (15-29 p.c.) of grafts was not recovered. This cannot have been due to heterozygosity in the mice, since independent isotransplants of skin in the inbred AKR and C3H mice of our colony have been uniformly successful. It is more likely that the majority represent surgical accidents, where adequate vascularisation through the capsule of the thymus graft was not achieved. However, the percentage of failed grafts rose with the age of the host, and graft failures may in part have been due to lower growth stimulation of the graft in the older hosts.

In a second series of grafts, one-week-old male C3H thymuses were grafted into groups of male C3H mice, aged either six weeks or six months. Although both groups of recipients were in the postinvolutional state, they differed in that the thymuses in six-week-old C3H mice normally lose 20 mg. in weight during the 6th-10th week age period, whereas the weight of the six-month-old

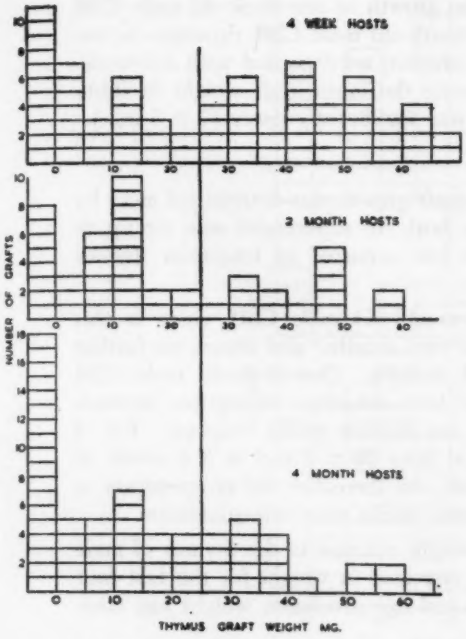

Fig. 3. Effect of the age of the host on the graft weight of one-week-old female AKR thymuses twenty-five days after grafting to female AKR recipients.

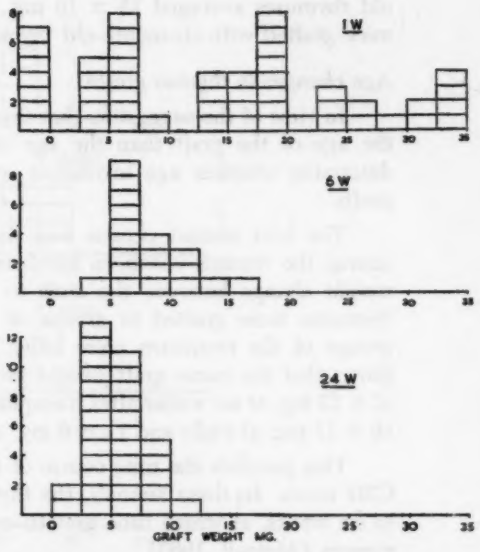

Fig. 4. Effect of the age of the grafted thymus on the graft weight twenty-five days after grafting to six-month-old male C3H mice.

C3H thymus is stable between 24-28 weeks of age (Metcalf, 1960). In nineteen, six-week-old hosts the average thymus graft weight was 16 ± 9 mg. In thirty, six-month-old hosts the average thymus graft weight was 15 ± 10 mg. There was thus no significant difference in the growth of the thymus grafts in these two types of postinvolutional host.

Effect of the age of the graft.

A more striking variation in the growth of thymus grafts was noted when the age of the recipient mice was kept constant, and the age of the mice donating the thymus grafts was varied.

Three groups of six-month-old male C3H mice were grafted with thymuses from male C3H mice aged either one week, six weeks or six months. Fig. 4 shows the weights of these thymus grafts twenty-five days after grafting. One-week-old thymuses grew significantly better (mean weight 16 ± 9 mg.) than six-week-old thymuses (mean weight 9 ± 3 mg.) or six-month-old thymuses (mean weight 6 ± 2 mg.). The differences between the latter two groups were slight, but significant statistically.

Similar results were obtained when the growth of one-week-old male C3H thymuses was compared with that of six-month-old male C3H thymuses in six-week-old male C3H hosts. The grafts in nineteen mice grafted with one-week-old thymuses averaged 15 ± 10 mg., whereas the mean graft weight in thirty mice grafted with six-month-old thymuses was significantly lower (7 ± 3 mg.).

Age changes in thymus grafts.

In view of the suggestion that thymus graft growth was determined more by the age of the graft than the age of the host, an experiment was set up to determine whether age involution weight loss occurred in long-term thymus grafts.

The host animal chosen was the six-month-old male C3H, since in this animal the thymus has been involuted for two months, and shows no further weight change between the sixth to ninth months. One-week-old male C3H thymuses were grafted to groups of these host animals. At regular intervals groups of the recipients were killed and the thymus grafts weighed. Fig. 5 shows that the mean graft weight increased from 16 ± 9 mg. at 3·5 weeks to 21 ± 13 mg. at six weeks after transplantation, and thereafter fell progressively to 16 ± 11 mg. at eight and 11 ± 6 mg. at twelve weeks after transplantation.

This parallels the time course of the weight changes in the thymus of male C3H mice. In these animals, the thymus increases in weight for the first four to six weeks, at which time growth ceases and age involution weight loss commences (Metcalf, 1960).

Growth of AKR and C3H thymuses in F_1 hybrid hosts.

One-week-old female AKR and C3H thymuses were grafted subcutaneously into groups of two-month-old female (AKR × C3H)F_1 hybrid mice. Twenty-five days after grafting some of the grafted animals were killed and the thymus grafts analysed cytologically. The remaining animals were killed and examined six weeks after grafting.

Table 1 presents the results of the cytological analyses of the AKR and C3H grafts in the F_1 hybrid hosts. All preparations were coded and counted as unknown preparations by a single observer. It will be seen that the AKR grafts analysed were slightly, but not significantly, heavier than the C3H grafts. The mitotic indices of the large and medium lymphocytes (combined) in the AKR grafts were significantly higher than those of C3H grafts. There were no differences in the percentages of the various types of lymphoid cells in these grafts.

In keeping with these differences in mitotic activity, AKR thymus grafts were found to be heavier than C3H grafts when examined six weeks after grafting. Fig. 6 presents these results. The mean graft weight in forty-three AKR grafts (30 ± 20 mg.) was more than twice that of forty C3H grafts (12 ± 10 mg.), a difference statistically significant at a level of $P < 0{\cdot}01$.

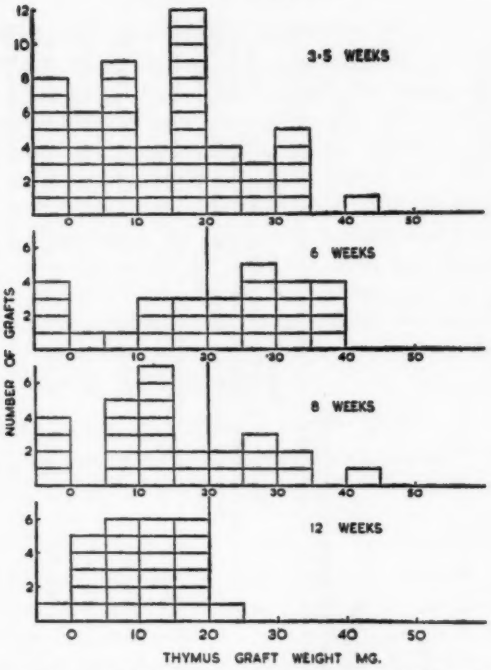

Fig. 5. Weight changes in 131 one-week-old male C3H thymus grafts at various intervals after grafting to six-month-old male C3H mice.

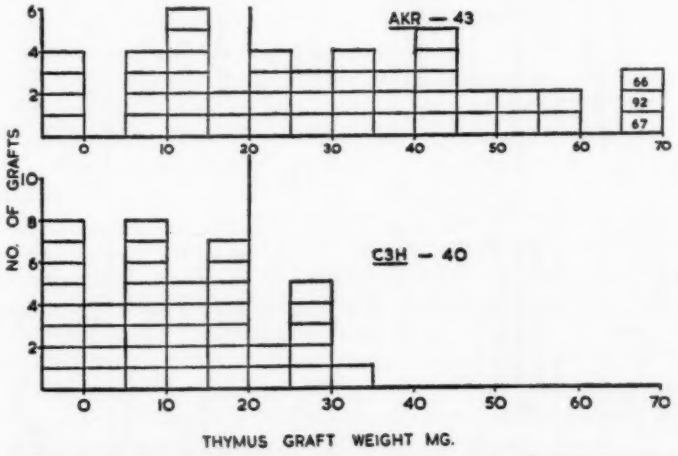

Fig. 6. Graft weights of one-week-old female AKR and C3H thymuses six weeks after grafting to two-month-old female (AKR × C3H)F_1 hybrid mice.

TABLE 1.

Cytological analysis of AKR and C$_3$H thymus grafts in F$_1$ hybrid hosts.

Strain of thymus graft	Number of grafts	Mean graft wt. mg.*	% Large lymphocytes	%Medium lymphocytes	%Small lymphocytes	%Mitotic cells	%Pyknotic cells	Mitotic index of primitive cells†
C3H	9	22±6	0·9±0·2	7·9±1·4	90·0±1·7	0·6±0·2	0·7±0·2	6·0±1·3
AKR	8	31±13	1·0±0·3	8·8±2·0	88·8±2·5	0·8±0·3	0·6±0·3	7·8±1·1‡

*±Standard deviation.
†Mitoses per 100 large and medium lymphoid cells (combined).
‡Significantly different $P < 0·01$.

TABLE 2.

Effect of thymus grafts on host lymphoid organ weights

Strain and age of donor mice	Strain and age of recipient mice at grafting	Number of mice	Mean body wt. gm.*	Mean spleen wt. mg.	Mean thymus wt. mg.	Mean mesenteric node wt. mg.	Mean inguinal lymph node wt. mg.	Mean opposite axillary nodes wt. mg.	Mean draining axillary nodes wt. mg.	Mean thymus graft wt. mg.
—	C3H♂ 6 months	30	37·5±4·0	89±7	28±10	42±10	21±3	22±3	22±3	—
C3H ♂ 1 week	C3H♂ 6 months	30	37·9±3·9	83±12	24±5	43±6	19±4	18±4	21±5	16±9
C3H♂ 6 weeks	C3H♂ 6 months	19	37·0±4·5	108±20	21±4	41±7	23±6	21±5	24±5	9±3
C3H♂ 6 months	C3H♂ 6 months	32	36·6±4·2	80±11	24±4	44±8	19±3	19±4	17±4	6±2
—	(AKRxC3H) F$_1$♀ 2 months	19	24·1±1·5	82±14	59±9	43±8	14±2	15±3	15±3	—
C3H♀ 1 week	(AKRxC3H) F$_1$♀ 2 months	33	23·4±2·5	89±12	62±8	42±6	14±3	14±2	16±3	22±11
AKR♀ 1 week	(AKRxC3H) F$_1$♀ 2 months	41	23·8±3·0	89±13	62±8	42±6	15±3	14±2	16±3	28±17

*±Standard deviation.

Lymphocyte levels in tail blood of thymus-grafted mice.

White cell levels in the tail blood were determined on twenty-four, six-month-old male C3H mice, grafted with whole, one-week-old C3H thymuses. Observations were made one, two and four weeks after grafting. Lymphocyte and polymorph levels in these mice showed no significant differences at any time from those of normal mice of the same strain and age, or from control mice, grafted subcutaneously with one-week-old C3H spleens.

In a separate experiment, the effect was investigated in six-week-old C3H mice, of grafting three one-week-old C3H thymuses into each recipient mouse. Eleven mice were grafted in this manner, and white cell levels were determined every two weeks. Observations were continued for five months after grafting. At no time did lymphocyte or polymorph levels deviate significantly from those of normal C3H mice of the same age and sex.

Lymphoid organ weights of mice bearing thymus grafts.

Mice bearing subcutaneous thymus grafts were routinely examined twenty-five days after grafting. At this time the weight of the graft and the weights of the various host lymphoid organs were determined.

Control mice were normal mice of the same age, strain and sex.

In the case of mice grafted with one-week-old thymuses, additional control groups consisted of mice grafted subcutaneously with spleens obtained from the same donor animals. This proved a useful control group, as the mean weights of the thymus and spleen in one-week-old C3H and AKR mice were found to be identical (18 mg.).

The host lymphoid organ weights were determined in over 800 mice bearing thymus grafts. In no group were the weights of any of the host lymphoid organs significantly different from those of the control mice. Table 2 shows a representative sample of these negative results.

When mice bearing thymus grafts were considered individually it was found that the weights of the two axillary nodes draining the graft site were usually heavier than the lymph nodes in the opposite axilla. Table 3 shows an analysis of the results obtained in mice, four weeks after the grafting of one-week-old thymuses and spleens. It will be noted that spleen grafts showed little tendency to increase the weight of the draining lymph nodes. In contrast, 60-75 p.c. of mice bearing thymus grafts had draining lymph nodes which were heavier than the opposite axillary lymph nodes. A notable exception to these findings was the result obtained when one-week-old AKR thymuses were grafted to four-week-old AKR mice. Six separate experiments of this type were performed, and in each group the results consistently showed the draining lymph nodes to be smaller than the opposite axillary nodes. This finding was the more remarkable since the thymus grafts in these animals were larger than in any other group, some grafts reaching a maximum weight of 75 mg.

TABLE 3.

Effect of thymus and spleen grafts on weight of draining lymph nodes.

Tissue grafted	Strain of donor	Age in weeks and sex of donor	Strain of recipient	Age in weeks and sex of recipient	Number of mice grafted	Percentage of mice with			Mean graft wt. mg.
						Dr.>Opp.	Opp.>Dr.	Dr.=Opp.	
Thymus	AKR	1F	AKR	16F	62	76	18	6	17·0
Thymus	AKR	1F	AKR	8F	47	65	19	15	19·0
Thymus	AKR	1F	AKR	4F	71	37	56	7	28·4
Thymus	AKR	1F	(AKRxC3H)F_1	8F	42	76	12	12	30·4
Thymus	C3H	1F	(AKRxC3H)F_1	8F	40	65	15	20	12·3
Thymus	C3H	1M	C3H	24M	35	66	29	5	13·7
Thymus	C3H	1M	C3H	6M	41	58	32	12	12·5
Thymus	AKR	1F	(AKRxC3H)F_1	8F	23	61	22	17	Failed grafts
Thymus	C3H	1M	C3H	24M	23	70	30	0	Failed grafts
Spleen	AKR	1F	AKR	16F	21	57	33	10	3·9
Spleen	AKR	1F	AKR	8F	15	40	27	33	10·7
Spleen	AKR	1F	AKR	4F	34	47	44	3	15·2
Spleen	C3H	1M	C3H	24M	29	52	28	20	6·0

TABLE 4.

Cytology of lymph nodes draining thymus graft site

Donor strain	Recipient strain	No. of mice	Tissue examined	%Large lymphocytes†	%Medium lymphocytes	%Small lymphocytes	Mitotic index*
C3H	(AKRxC3H)F_1	4	Draining lymph nodes	1·4±0·3	7·4±1·2	91·4±1·7	0·8±0·1
		4	Opposite lymph nodes	1·1±0·6	6·8±0·7	92·3±1·2	0·8±0·3
AKR	(AKRxC3H)F_1	4	Draining lymph nodes	1·1±0·6	6·8±0·4	91·4±2·0	0·8±0·1
		4	Opposite lymph nodes	0·6±0·2	5·4±1·1	93·6±1·0	0·7±0·2

†±Standard deviation.
*Mitoses per 100 large and medium cells (combined).

No correlation was found in individual mice between the size of the thymus graft and either the absolute weight of the draining lymph nodes, or the degree to which the weight of the draining lymph nodes exceeded that of the opposite lymph nodes. It will also be observed in the Table that where mice were grafted with thymuses, but at autopsy no graft could be identified, draining lymph nodes were also heavier than the opposite nodes.

The nature of the weight increase in the draining lymph nodes of mice with thymus grafts was investigated in more detail by carrying out a cytological analysis of draining and opposite axillary lymph nodes. Single cell suspensions of the nodes were prepared and an analysis was made of the percentage of the various lymphoid cell types and the mitotic indices of the primitive lymphoid cells. Table 4 shows the results obtained from an analysis of the lymph nodes from two-month-old female (AKR × C3H)F_1 hybrid mice, grafted four weeks previously with one-week-old C3H or AKR thymuses. No significant changes were seen in the draining lymph nodes, in the percentages of large, medium and small lymphocytes, or in the mitotic indices of the primitive cells.

Histologically, the changes in the lymph nodes draining thymus grafts proved difficult to interpret, because of the normal variability in the architecture of these nodes. Some draining lymph nodes were seen in which the lymph sinuses were packed with small lymphocytes. Other nodes exhibited hyperplasia of the reticuloendothelial elements, primarily in the medullary region of the nodes. However, similar changes were frequent in the control nodes. In agreement with the negative cytological data, germinal centres did not appear to be larger, more numerous, or more active in the draining lymph nodes than in the opposite axillary nodes.

A cytological analysis was also made of host thymus tissue in mice bearing thymus grafts. Six six-month-old male C3H mice were grafted with one-week-old C3H thymuses. Six control mice were grafted with one-week-old C3H spleens. Four weeks later the host thymuses were removed and examined cytologically. No differences in the thymus cytology were found between the two groups and in neither group did the glands differ significantly from normal seven-month C3H thymuses.

DISCUSSION.

In the present experiments isologous thymus graft tissue has been found to increase in weight in animals whose own thymuses were either increasing in weight, stable in weight or rapidly losing weight, during the same observation period. This suggests that the growth of such thymus grafts is little influenced by humoral regulators of lymphopoiesis circulating in the host. However, thymus grafts grew to a slightly larger size in preinvolutional hosts than in post-involutional hosts, indicating that general regulators of lymphopoiesis may not be entirely without effect on such grafts.

The size attained by thymus grafts was chiefly determined by the age of the thymus grafted – the younger the thymus, the larger the size attained. Thymus age involution has been shown to be associated with and probably caused by a sharp fall in the mitotic activity of the cortical lymphoid cells (Nakamura and Metcalf, 1961). In the present experiments preinvolutional thymuses grew to a larger size than postinvolutional thymuses. Further, one-week-old male C3H grafts in six-month-old hosts underwent weight increase followed by weight loss in the same time sequence as thymus weight increase and subsequent involution occur in intact male C3H mice. These results suggest strongly that the proliferative activity of thymus tissue on grafting is largely autonomous with respect to general body regulators of lymphopoiesis and is primarily determined by stimuli arising from within the thymus itself. By inference the same conclusion may be applicable to the nature of the regulation of lymphopoiesis in the thymus *in situ*. The constant presence of epithelial cells in the centre of the lymphoid cells of the thymus lobes and the small islands in thymus graft tissue suggests some functional inter-relationship between these two cell types. The nature of this inter-relationship remains unknown.

The present findings are in disagreement with the statements by Rask-Nielsen and Andreasen (1955), made on the basis of very few observations, that with thymus grafts the age of the donor was of no significance and that the age of the recipient determined the degree of age involution of the implant.

In the uniform environment of the F_1 host, AKR and C3H thymus grafts maintained the growth differences previously noted between the thymuses of mice of these strains. It has been demonstrated by Law (1952) and more recently by Miller (1961), that parental thymus grafts in F_1 hybrid mice are frequently repopulated entirely by host F_1 hybrid lymphoid cells. If this be the case, the greater size and proliferative activity of AKR grafts may be due not to an inherently greater proliferative capacity of AKR lymphoid cells, but to stronger stimuli emanating from non-lymphoid elements within the AKR thymus, driving the repopulating F_1 or AKR lymphoid cells to higher proliferative activity.

The persistence of parental strain differences in the proliferative activity of thymic tissue, after grafting, may explain in part the difference in lymphoid leukaemia incidence in thymectomised F_1 hybrid mice, grafted with high- and low-leukaemia strain thymuses (Law, 1952).

Grégoire and Duchateau (1956) have reported that rabbit or rat thymus grafts in rats produced a significant increase in weight and germinal centre activity in the host lymph nodes, particularly in those draining the graft site. However, their experiments were conducted on very small numbers of animals, which were also not inbred.

In the present experiments the thymus grafts in many animals attained a size larger than that of the host thymus, and exhibited high, thymic-type mitotic activity. Despite this, virtually no effects were detectable on the lymphoid

tissues of the host animals. Calculation of the lymphocyte production in the graft and the host animal, based on direct mitotic counts, suggested that some grafts were producing half as many lymphocytes as were being produced in the entire host animal. The failure of thymus graft lymphocyte production of this magnitude to affect the mass of the host lymphoid organs may be due to the failure of graft lymphocytes to enter the host circulation or to an increased utilisation and destruction of lymphocytes by the host.

The significance of the slight weight increase in the lymph nodes draining the graft site is difficult to assess. No evidence was obtained of any stimulation of mitotic activity of the lymphoid elements of these nodes, and the weight increase could well be a non-specific response to operative trauma and to the presence of actively growing tissue in the graft site.

It is noteworthy in the present experiments that no lymphoid involution occurred in F_1 hybrid mice, grafted with parental thymuses. This is a situation in which, theoretically, homologous disease with lymphoid involution should occur. However, the present observations are in agreement with Dubert and Kaplan (1961) who found that, whereas parental thymus cell suspensions produced homologous disease when injected into F_1 hybrid mice, thymus grafts had no such effect.

REFERENCES.

Carnes, W. H., Kaplan, H. S., Brown, M. B., and Hirsch, B. B. (1956): Cancer Res., 16, p. 429.
Dubert, A., and Kaplan, H. S. (1961): Fed. Proc., 20, p. 40.
Grégoire, C., and Duchateau, G. (1956): Archives Biol., 67, p. 269.
Law, L. W. (1952): J. Natl. Canc. Instit., 12, p. 789.
McEndy, D. P., Boon, M. C., and Furth, J. (1944): Cancer Res., 4, p. 377.
Metcalf, D. (1960): *Ibid.*, 20, p. 1347.
Miller, J. F. A. P. (1960): Brit. J. Cancer, 14, p. 93.
Miller, J. F. A. P. (1961): In C.I.B.A. Symposium "Murine Tumour Viruses", Churchill, London (in press).
Nakamura, K., and Metcalf, D. (1961): Brit. J. Cancer, 15, p. 306.
Rask-Nielsen, R., and Andreasen, E. (1955): Acta path. microbiol. Scand., 36, p. 1.

BIURET REACTIVE MATERIALS IN SEMEN

by R. G. WALES, T. W. SCOTT AND I. G. WHITE

(From the Department of Veterinary Physiology, University of Sydney, Sydney, Australia).

(*Accepted for publication 25th August, 1961.*)

SUMMARY.

A satisfactory method of estimating biuret reactive material in semen is described. Human, ram, bull, fowl and dog semen vary in protein, proteose and peptide content. Values for biuret reactive material range from 1·80 gm./100 ml. in fowl semen to 7·32 gm./100 ml. in bull semen. Proteolysis occurred in human semen during incubation but was not detected in the semen of other species studied.

Considerable species differences occurred in the amount of protein precipitated on heating semen at pH 4·6.

INTRODUCTION.

Since Autenrieth (1917) first used the biuret reaction for determining protein in blood serum, urine and ascitic fluid this method has been extensively studied (Hiller, 1927; Fine, 1935) and simplified by the introduction of a copper reagent stabilised with sodium potassium tartrate and potassium iodide (Weichselbaum, 1946; Gornall, Bardawill and David, 1949). Further investigations seemed warranted, particularly in relation to the estimation of the protein content of semen.

Most protein analyses of semen have hitherto been based on the rather tedious estimation of nitrogen by the Kjeldahl technique and it was felt that the biuret reaction might meet the need for a simple, rapid and accurate method. Estimation by the biuret method of protein in whole semen, as opposed to the seminal plasma, requires a procedure which will digest the spermatozoa and give clear solutions for reading in the spectrophotometer.

Larson and Salisbury (1954) have indicated that 90 p.c. of the total nitrogen present in bull seminal plasma is protein in nature and non-dialysable; the majority of it is also heat-coagulable. Lundquist (1952) and Jacobsson (1950) have reported that the proteins in human semen undergo hydrolysis after ejaculation. In view of these findings studies on the precipitable and non-precipitable biuret reactive material in the semen of various animals have been undertaken after incubation and heating.

MATERIALS AND METHODS.

Semen.

Semen samples were frozen in dry ice immediately after ejaculation and maintained at −79° C. until required for the analyses, which were usually performed within a few hours of collection. Tests showed that no change occurred in the protein levels of deep frozen specimens. Thus, the mean protein concentration of ejaculates analysed immediately and again after three days at −79° C. were 4·69 p.c. and 4·77 p.c. respectively, and did not differ significantly ($t_{(28)} = 0{\cdot}97$, $P > 0{\cdot}05$).

Where incubated samples have been compared with unincubated controls, the ejaculate was divided into two on collection and half of it was deep frozen. After incubating the remainder at 37° C., estimations were simultaneously made on both incubated and unincubated samples.

Protein precipitation.

The protein precipitants used were 20 p.c. trichloroacetic acid (2 vols.), redistilled ethanol (6 vols.) and redistilled acetone (6 vols.). After thorough mixing and centrifuging the supernatant was removed and the tubes inverted to drain. The precipitates were not washed as tests with ram semen showed that the protein values obtained for washed and unwashed trichloroacetic acid precipitates did not differ significantly (4·90 gm./100 ml. and 4·81 gm./100 ml. respectively; $t_{(24)} = 1{\cdot}84$, $P > 0{\cdot}05$). Only the biuret reactive material in semen precipitated by trichloroacetic acid, ethanol or acetone is referred to as protein.

Reagents and procedure.

The procedure for the experiments was based on the recommendations of Kirk (1950) and modified to give optimum conditions for seminal analysis. Preliminary experiments showed that digestion of ram semen with 2 or 4N NaOH at 40° or 60° C. disintegrated the cells and increased the colour developed in the biuret reaction. Although 40° C. is as effective as 60° C. over a 15-minute digestion period the time can be considerably reduced if the higher temperature is used.

Thus, to obtain a reliable estimate of the total protein in ram semen it is necessary to precipitate the protein and digest it with 2N NaOH. The following procedure is recommended. An appropriate volume of semen, e.g. 0·1 ml., is placed in a centrifuge tube graduated at 12·5 ml. and ethanol (0·6 ml.) or trichloroacetic acid (0·2 ml.) added. After centrifuging, the tubes are inverted to drain and the precipitate mixed with 0·5 ml. of 2 N NaOH and placed in a water bath at 60° C. until clear. A small volume of 0·8N NaOH is added, followed by 2 ml. of copper reagent and the solution made up to the mark with 0·8N NaOH. After incubating at 37° C. for 30 min., the optical density is read at 540 mμ.

In all cases, standard calibration curves were constructed for each group of unknowns. A slight but statistically significant decrease in optical density of the standards was found after 3 hr.; solutions were therefore read soon after colour development.

Kjeldahl nitrogen.

Total nitrogen was estimated by the micro-Kjeldahl method. Samples containing about 1 mg. of nitrogen were digested for 6 hr. with potassium sulphate, sulphuric acid and mercuric sulphate as catalysts (Hiller, Plazin and Van Slyke, 1948). The distillation was carried out in a Parnas-Wagner apparatus (Parnas and Wagner, 1921) and 25 ml. of distillate collected in a flask containing 10 ml. of indicator ($6{\cdot}6 \times 10^{-4}$ p.c. methyl red and $3{\cdot}3 \times 10^{-4}$ p.c. bromcresol green in 2 p.c. boric acid).

Statistical analysis.

Factorial design has been used throughout because it provides an efficient and comprehensive method for isolating main effects and their interactions. All results have been subjected to standard analyses of variance which are presented in summary form giving only degrees of freedom (D.F.) and variance ratios (V.R.) for each source of variation. The residual variances are given at the base of the variance ratio columns and asterisks are used to denote significant differences. Each experiment has been repeated with several different ejaculates and these are referred to as samples or replicates in the analyses of variance.

RESULTS.

Estimation of protein in ram semen.

Table 1 shows the effect of three common protein precipitants, viz. trichloroacetic acid, ethanol and acetone, on the level of biuret reactive material in ram semen. Duplicate estimations were made on undigested precipitates and on precipitates digested with three volumes of 2 N NaOH for 15 min. at 60° C. The undigested samples had to be cleared by centrifuging before reading in the spectrophotometer. The digested samples, however, were clear and centrifugation was unnecessary. The three protein precipitants gave similar results and in each case some biuret reactive material was left in the supernatant. Checks showed that complete precipitation of biuret reactive material occurred with egg albumin. When seminal plasma obtained by centrifuging freshly col-

TABLE 1.

A comparison of the biuret reactive material (expressed as gm./100 ml.) in ram semen and in the precipitate obtained by treatment with deproteinising agents. The means of duplicate estimations on each of three semen samples are given.

Protein precipitant	Undigested	NaOH digested	Means
Nil	5·32	5·66	5·49
T.C.A.	4·97	5·04	5·00
Ethanol	4·86	5·10	4·98
Acetone	4·80	5·30	5·05
Means	4·99	5·28	

Summary of the analysis of variance.

Source of variation	D.F.	Variance ratio
Effect of precipitation		
Pptd. v. unpptd. controls	1	22·86**
Within precipitations	2	0·18
Effect of NaOH digestion	1	11·08**
Precipitation x digestion	3	1·00
Sample differences	2	41·83**
Sample interactions	14	0·47
Duplicate variance (error)	24	0·0908

** $P<0·01$.

lected whole semen was used, the precipitation of biuret reactive material was again incomplete but digestion did not increase the values (Table 2).

To compare the biuret and micro-Kjeldahl methods, five estimations on each whole ejaculate were made following trichloroacetic acid precipitation. The mean protein values found for 5 ejaculates were 4·69 p.c. and 4·51 p.c. respectively. The difference is not significant ($t_{(32)} = 1·38$, $P > 0·05$) although there was some variation from ejaculate to ejaculate.

TABLE 2.

A comparison of the biuret reactive material (gm./100 ml.) in seminal plasma and in the precipitate obtained by treatment with 2 volumes of 20 p.c. trichloroacetic acid. The values are the means of duplicate estimations for each of four plasma samples.

Protein precipitant	Undigested	NaOH digested	Mean
Nil	3·23	3·32	3·28
T.C.A.	3·07	2·92	3·00
Means	3·15	3·12	

Summary of the analysis of variance.

Source of variation	D.F.	Variance ratio
Effect of precipitation	1	50·70**
Effect of digestion	1	0·65
Precipitation x digestion	1	5·86*
Sample differences	3	27·07**
Sample interactions	9	0·98
Duplicate variance (error)	16	0·125

* $P<0·05$. ** $P<0·01$.

Effect of incubating semen.

The biuret reactive material in the semen and seminal plasma of man, ram, bull, fowl and dog before and after incubation for 3 hr. is given in Table 3. Trichloroacetic acid and ethanol were used as precipitants in each case. Since the residual variances for whole semen were significantly greater than those found for seminal plasma ($F_{(100,100)} = 2·29$, $P < 0·01$) separate analyses for semen and seminal plasma were made for each species.

Human. There was a considerable decrease in the protein content of both whole semen and the seminal plasma on incubation. The decrease in total biuret reactive material, however, was considerably less, indicating that not all of the hydrolysed protein had been broken down to free amino acids. Prior to incubation almost all of the biuret reactive material could be precipitated by trichloroacetic acid or ethanol; after incubation only a fraction of this could be precipitated.

TABLE 3.

The biuret reactive material (gm./100 ml.) in the semen and seminal plasma of various animals. The samples were analysed before and after three hours' incubation at 37° C. and the values in the table are the mean of five replicates for each species.

Sample	Time	Extract	Human	Ram	Bull	Fowl	†Dog
Semen	Zero	Whole semen	4·00	5·16	7·32	1·80	2·36
		TCA ppt.	4·07	4·99	6·80	2·00	2·35
		Ethanol ppt.	3·82	4·87	7·31	1·79	2·31
	3 hr	Whole semen	3·62	5·07	7·42	1·82	2·61
		TCA ppt.	3·23	5·10	6·59	1·99	2·49
		Ethanol ppt.	3·12	4·70	7·24	1·71	2·58
Seminal plasma	Zero	Whole plasma	4·07	3·51	7·04	0·93	2·15
		TCA ppt.	3·80	3·24	6·24	0·86	2·06
		Ethanol ppt.	4·07	3·35	6·85	0·88	2·16
	3 hr	Whole plasma	3·68	3·51	6·91	0·86	2·24
		TCA ppt.	3·04	3·14	6·42	0·76	2·09
		Ethanol ppt.	3·39	2·99	6·90	0·79	2·12

† Semen = prostatic fluid + spermatozoa bearing fraction of ejaculate. Seminal plasma = prostatic fluid.

Summary of the analyses of variance.

Source of variation	D.F.	Variance ratios									
		Semen					Plasma				
		Human	Ram	Bull	Fowl	Dog	Human	Ram	Bull	Fowl	Dog
Effect of incubation	1	39·22**	0·13	0·24	0·55	12·71**	86·26**	5·20**	0·12	6·36*	0·54
Effect of precipitation											
Whole semen v ethanol pptn.	1	7·45*	4·42*	0·41	1·35	0·34	3·06	16·81**	0·83	1·79	1·17
TCA v rest	1	0·00	0·48	23·32**	23·04**	0·51	30·72**	4·21	37·64**	1·00	5·23*
Incubation/precipitation											
Incubation/overall pptn.	1	2·93	0·05	0·86	0·69	0·21	5·59*	2·38	1·69	0·33	1·15
Incubation/TCA v ethanol	1	0·97	0·84	0·19	0·31	0·82	0·25	2·34	0·37	0·34	0·66
Replicates	4	9·05**	36·53**	46·47**	71·73**	138·56**	174·76**	21·22**	88·86**	21·39**	796·95**
Residual	20	0·078	0·123	0·114	0·012	0·029	0·032	0·036	0·063	0·011	0·011

* $P < 0·05$. ** $P < 0·01$.

Ram and bull. There was little breakdown in protein or decrease in the total biuret reactive material on incubation. As before, not all of the biuret reactive material in ram semen was precipitated by ethanol or trichloroacetic acid. With the bull, however, ethanol caused complete precipitation.

Fowl. There was a slight decrease in the protein and total biuret reactive material on incubating the seminal plasma. Most of the biuret reactive material in seminal plasma was precipitated by trichloroacetic acid or ethanol.

Dog. No decrease in the protein or biuret reactive material occurred on incubating prostatic fluid. If anything, there was a slight increase in both when prostatic fluid and spermatozoa were incubated together. The biuret reactive material was completely precipitated by ethanol and trichloroacetic acid.

The effect of 3 hr. incubation on the biuret reactive material in human semen is further shown in Table 4. Heating the unincubated seminal plasma for 1 hr. after the pH had been adjusted to 4·6 by the addition of 5 volumes of 1 M acetate buffer (Larson and Salisbury, 1954) caused little biuret material to be precipitated. Trichloroacetic acid precipitated 55 p.c. of the biuret reactive material, leaving about 35 p.c. in the supernatant. Incubation decreased the total amount of biuret material in the untreated plasma by 7 p.c.; trichloroacetic acid precipitated less and more was left in the supernatant. Since trichloroacetic acid precipitated 95 p.c. of the biuret material in unheated samples (Table 3) heating must decrease the amount of protein precipitated by trichloroacetic acid.

Similar tests were made to determine the distribution of biuret material in bull seminal plasma (Table 4). One hour at 100° C. (pH 4·6) precipitated 50 p.c. of the material and a further 45 p.c. was precipitated by trichloroacetic acid. When the three fractions of dog semen were heated at pH 7 for 10 min. at 100° C. most of the biuret reactive material was precipitated (Table 5). Heating human and bull seminal plasma at pH 7 caused no precipitation.

TABLE 4.

The distribution of biuret reactive material (gm./100 ml.) in human and bull seminal plasma.

Sample analysed	Human				Bull unincubated	
	Unincubated	Incubated	Mean difference	t	Mean	S.D.
A. Untreated plasma	4·58	4·31	0·27	4·5*	7·59	0·24
B. Ppt. after boiling at pH 4·6	0·55	0·86	0·31	4·3*	3·90	1·27
C. T.C.A. ppt. of supernatant from B	2·54	1·85	0·69	1·7	3·40	0·49
D. Supernatant from C	1·56	1·96	0·40	5·0*	—	—

* $P<0·05$.

TABLE 5.

The amount of biuret reactive material (gm./100 ml.) in dog semen. Mean values ± standard errors for eight samples are given.

Sample	Semen fraction		
	Presperm	Sperm	Prostatic
Unheated	0·91±0·02	4·94±0·49	3·26±0·42
Supernatant after heating	0·17±0·02	0·50±0·10	0·07±0·01

DISCUSSION.

A large proportion of the protein-like material in fresh human semen passes through semipermeable membranes but is not heat coagulable and, although precipitated by ethanol, is not precipitated by trichloroacetic acid. This material has been classified as propeptone, hemialbumose, primary proteose and secondary proteose (Mann, 1954).

The results obtained with the various protein precipitates suggest that human and bull semen contain proteoses, but no peptides. Although it has been reported that a large proportion of protein-like material in human semen is not precipitated by trichloroacetic acid (Goldblatt, 1935), over 90 p.c. of the biuret reactive material was precipitated by trichloroacetic acid in the present study. Ram semen, on the other hand, contains peptides, but no proteoses. All the biuret reactive material in dog and fowl semen, however, was protein in nature.

In the incubation experiments (Table 3) the experimental error for whole semen was larger than that for seminal plasma. The heterogeneous nature of whole semen, particularly of human samples, increases the difficulty of pipetting small quantities accurately. In addition, the necessity to digest the spermatozoa with NaOH may be another factor contributing to the higher experimental error.

As reported by Lundquist (1952) and Jacobsson (1950), breakdown of protein in human semen occurred during incubation, but in the other species examined there was little evidence of this. However, Bhargava (1957) and Bhargava, Bishop and Work (1959a), have been able to demonstrate some proteolysis in bull semen using the more sensitive method of measuring an increase in amino nitrogen. Incubating dog spermatozoa with prostatic fluid caused an increase in biuret reactive material. This might indicate that dog spermatozoa, like those of the bull (Bhargava, Bishop and Work, 1959b), can synthesise protein.

There are considerable species differences in the extent to which proteins are precipitated by heat. Heating dog semen near neutrality causes most of the protein to coagulate. The results for human and bull semen confirm earlier

reports (Huggins, Scott and Heinen, 1942; Larson and Salisbury, 1954); thus, human semen was not coagulated by heat, but about 50 p.c. of the protein in bull semen was precipitated after heating at pH 4·6.

Acknowledgments. The authors are indebted to Professor C. W. Emmens for interest and advice. The work was performed under a grant from the Planned Parenthood Federation of America.

REFERENCES.

Autenrieth, W. (1917): Munch. Med. Woch., 64, p. 241. Cited by Robinson, H. W., and Hogden, C. G. (1940): J. biol. Chem., 135, p. 707.
Bhargava, P. M. (1957): Nature, 179, p. 1120.
Bhargava, P. M., Bishop, M. W. H., and Work, T. S. (1959a): Biochem. J., 73, p. 242.
Bhargava, P. M., Bishop, M. W. H., and Work, T. S. (1959b): *Ibid.*, 73, p. 247.
Fine, J. (1935): *Ibid.*, 29, p. 799.
Goldblatt, M. W. (1935): *Ibid.*, 29, p. 1346.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949): J. biol. Chem., 177, p. 751.
Hiller, A. (1927): Proc. Soc. exp. Biol., 24, p. 385.
Hiller, A., Plazin, J., and Van Slyke, D. D. (1948): J. biol. Chem., 176, p. 1401.
Huggins, C., Scott, W. W., and Heinen, J. H. (1942): Amer. J. Physiol., 136, p. 467.
Jacobsson, L. (1950): Acta physiol. Scand., 20, p. 88.
Kirk, P. L. (1950): "Quantitative Ultramicroanalysis." New York: John Wiley.
Larson, B. L., and Salisbury, G. W. (1954): J. biol. Chem., 206, p. 741.
Lundquist, F. (1952): Acta physiol. Scand., 25, p. 178.
Mann, T. (1954): "The Biochemistry of Semen." London: Methuen.
Parnas, J. K., and Wagner, R. (1921): Biochem. Z., 125, p. 253.
Weichselbaum, T. E. (1946): Am. J. clin. Path. tech. Sci., 10, p. 40.

THE LIFE CYCLE OF *DERMATOPHILUS DERMATONOMUS*, THE CAUSAL AGENT OF OVINE MYCOTIC DERMATITIS

by D. S. ROBERTS

(From the Division of Animal Health, McMaster Laboratory, C.S.I.R.O., Glebe, N.S.W., Australia).

(*Accepted for publication 30th August, 1961.*)

SUMMARY.

An account is given of the life cycle of *Dermatophilus dermatonomus* as observed in cultures and in lesions of mycotic dermatitis. Factors which were shown to govern the stages of development in cultures, appear to have a similar influence within the lesion.

It is suggested that the sheep's skin may be the only natural habitat for *D. dermatonomus* and that survival of the species may therefore depend on the repeated transmission of infection.

INTRODUCTION.

Mycotic dermatitis is caused by closely related varieties or species of an actinomycete which invades the cornifying layer of skin and provokes the formation of crusts or scabs. The disease occurs naturally in sheep, cattle, horses and goats.

The earliest available record of mycotic dermatitis is that of van Saceghem (1915) who described a disease of cattle in the Belgian Congo, which he called *dermatose contagieuse* (*impetigo contagieux*). Van Saceghem found the lesions to contain a branching filamentous organism which divided both transversely and longitudinally to form spores. He named it *Dermatophilus congolensis*. In the following year van Saceghem (1916) isolated the organism in culture, where he noted its morphological similarity to the hyphae he had seen in material from lesions.

The first account of mycotic dermatitis in sheep was by Bekker (1928) in South Africa, who described an exudative condition of the woolled skin, with the formation in the fleece of hard pyramidal scabby masses. He called the disease "lumpy wool", and mentioned that it seemed to be more prevalent in wet seasons. The fungus-like form of the causal organism was discovered by Seddon as reported by Henry (1928). Seddon (1929) showed that the disease was transmissible when he reproduced it by the application of scab material to the skin of sheep.

The ovine mycotic dermatitis organism was first isolated by Bull (1929). He described its characteristics in lesions and cultures and named it *Actinomyces*

dermatonomus. His description corresponded closely to that given by van Saceghem for the organism isolated from cattle. Bull demonstrated the specificity of the infection by reproducing mycotic dermatitis in sheep with pure cultures of *A. dermatonomus*. Seddon (1929) and Bull (1929) both described the superficial nature of the invasion into the skin, and the exudative inflammatory response.

Austwick (1958) has reclassified the mycotic dermatitis organisms, placing them in a new family, *Dermatophilaceae*, of the Order, *Actinomycetales*, with a single genus, *Dermatophilus*. He lists three species, *D. congolensis* van Saceghem from bovine streptothricosis, *D. dermatonomus* (Bull) from ovine mycotic dermatitis, and *D. pedis* (Thompson and Bisset) from strawberry footrot of sheep. His classification takes into account the important differences between these organisms and the other actinomycetes, and also the priority of van Saceghem's generic name *Dermatophilus*.

The transmission of infection has been discussed by several workers. Bull (1929) suggested that the causal organism was a saprophyte which grew in the fleece under wet conditions and then penetrated the skin. Seddon (1929 and 1953), on the other hand, considered that the soil probably became contaminated by scabs shed from infected sheep and that the organism was then carried in dust into the fleece where, in wet weather, it grew and invaded the skin in the manner proposed by Bull. An association of infection with wetting of the skin was suggested by Harris and Harding (1950) and by Austwick and Davies (1958). Austwick and Davies thought that skin damage by wetting might facilitate invasion.

Moule and Sutherland (1947) and Seddon (1953) indicated that mycotic dermatitis in cattle was associated with skin damage by abrasion, and not necessarily with wet weather. Seddon considered that lambs, likewise, may become infected by rubbing against affected mothers. He also suggested that the amniotic fluid on their coats at birth would permit the organism to grow and invade the skin during the first days of post-natal life.

Several recent contributions to the biology of *Dermatophilus* spp. appear to have a more fundamental bearing on the process of transmission. In reporting the initial isolation of *D. pedis*, Thompson (1954) described what is now accepted (Austwick, 1958) as the key distinguishing feature of the genus, the transverse and longitudinal division of the hypha to form motile cocci, which have been called zoospores (Austwick, 1958). The development of such motile forms in cultures of *D. dermatonomus* was first observed by Simmons (1955). Roberts (1957a and b) discussed the probable function of the zoospore as the infecting agent in the transmission of lumpy wool, and showed that the extension of the mycelium on the one hand and its internal division to form spores, on the other, were favoured by strikingly different cultural conditions. Hyphae developed most readily in a cultural environment corresponding in many respects to the environment which invading hyphae would be expected to en-

counter in the depths of the lesion. The formation of motile spores was precipitated by a reversal of the conditions, that is, by an increase in air supply and by falls in both nutrient supply and temperature. Roberts suggested that such conditions were likely to be found at the surface of the lesion, where zoospores could therefore develop and be released to the exterior. This was supported by the observation that, in scab material, hyphae could only rarely be found whereas coccoid forms were remarkably numerous.

Thompson (1954) showed that suspensions of either spores or filaments of *D. pedis* were infective when applied to the skin, and Roberts (1957a) was able to infect scarified skin with *D. dermatonomus* zoospores. Because of the motility of the spores and their more manoeuvrable size and shape compared with hyphae, and because the organism proved very difficult to grow in the presence of other aerobes, Roberts (1957b) deduced that there was probably no multiplication outside the sheep's skin and that new lesions were initiated by the invasion of spores which had come from existing lesions.

The observations reported in this paper form the basis for studies of the relationship between *Dermatophilus dermatonomus* and its host, and for an investigation of its fate outside the host.

Materials and Methods.

Strains of D. dermatonomus. The strains studied were isolated from mycotic dermatitis lesions on sheep which had become naturally infected in the field.

Media: *Broth medium*: 0·5 p.c. Oxoid Lab-Lemco beef extract, 1·0 p.c. Difco Proteose Peptone, 0·1 p.c. Difco yeast extract, 0·15 p.c. glucose and 0·5 p.c. sodium chloride were dissolved in tap water. The pH was 7·2 after autoclaving. *Agar medium*: This was prepared by the addition of 1·5 p.c. Difco agar to the broth medium.

Histological examination of lesions. Biopsy specimens of both natural and artificially induced lesions were fixed, sectioned and stained by either the Gram method or with haematoxylin and eosin. Lesions were induced by the application of spores from broth cultures, to patches of woolled skin which had been clipped and then defatted by washing gently with "Shell X-4" light petroleum.

Immersion refractometry. This was performed by means of a phase contrast microscope, according to the method of Barer and Joseph (1954, 1955a and b) and Barer (1956). Solutions of Bovine Plasma Albumin Fraction V (Armour and Company) were used as immersion media. Protoplasmic solids concentration was calculated from the refractive index, as described by Barer and Joseph.

RESULTS.

The life cycle, and factors which influence it in vitro.

Germination of the spore.

The zoospores are ovoid cells. The mean diameter for different strains ranges from about 0·5μ to about 1·1μ. They are illustrated in Fig. 1. They are highly motile and have been seen to travel several millimetres in one minute in liquid media. The spores remain reproductively dormant in the absence of an adequate stimulus to germinate. The stimulus appears to be the

LEGENDS TO FIGURES

Fig. 1. Free ungerminated zoospores, suspended in a 26 p.c. albumin solution. They are darker than the background showing that their protoplasm has a higher solids content, or lower water content, than the medium. (Phase contrast; magnification 1,600 x.)

Fig. 2. Germination and budding after 5 hours' incubation. The first transverse septum is already present in one hypha. The protoplasm has become paler than in Fig. 1 and now matches the medium (26 p.c. albumin). This indicates a rise in water content. Note the increased diameter of the original spores. (Phase contrast; magnification 1,600 x.)

Fig. 3. Hyphae one day old, suspended in 26 p.c. albumin. Note the lightness of contrast in the undivided growing tips, because of their high water content. The fall in water content accompanying septation is seen as a darkening of the protoplasm in several hyphae. (Phase contrast; magnification 1,600 x.)

Fig. 4. A branching hypha, one day old. Each branch has apparently been formed by the budding of a segment of the hypha. (Phase contrast; magnification 1,600 x.)

Fig. 5. The initial transverse septation is complete in these 2-day-old hyphae. The segments are shorter than they are wide and are bulging laterally. The first longitudinal septa have formed in a number of segments. A capsule which has begun to develop on the hyphae may be seen as an irregular layer of material which is light in contrast. (Phase contrast; magnification 1,600 x.)

Fig. 6. Further binary fission, with the formation of cocci, in a hypha one day old. (Phase contrast; magnification 1,600 x.)

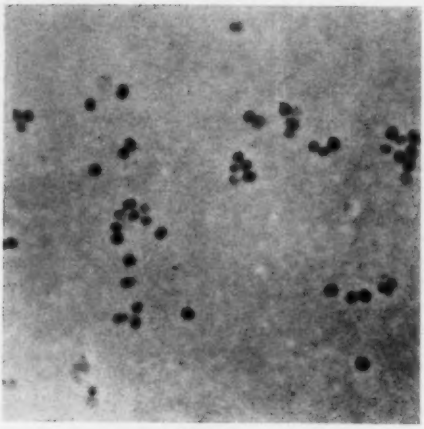

Fig. 1.

Fig. 2.

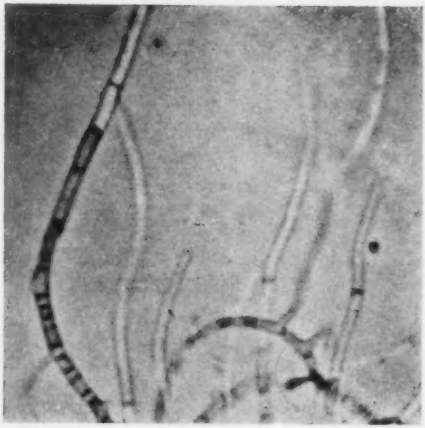

Fig. 3.

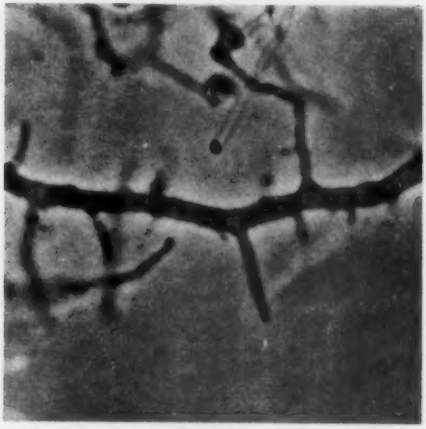

Fig. 4.

Fig. 5.

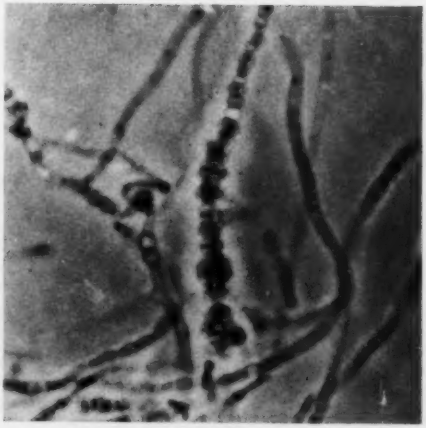

Fig. 6.

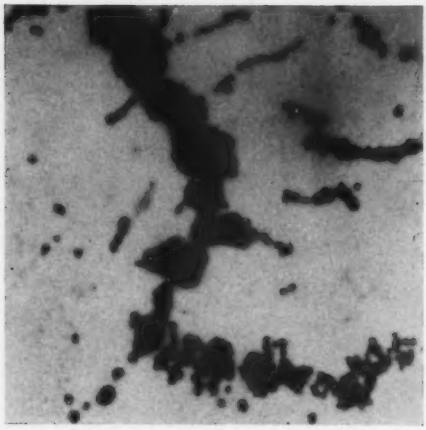

Fig. 7.

Fig. 8.

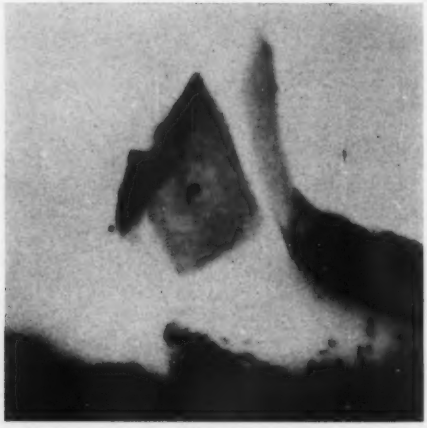

Fig. 9.

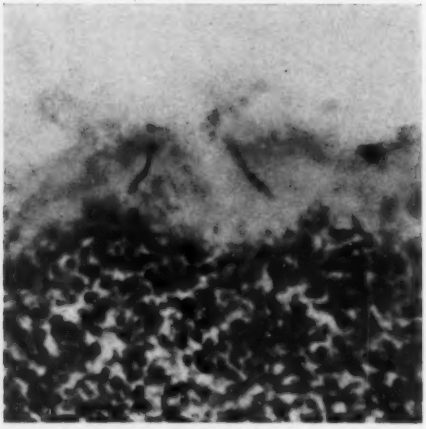

Fig. 10.

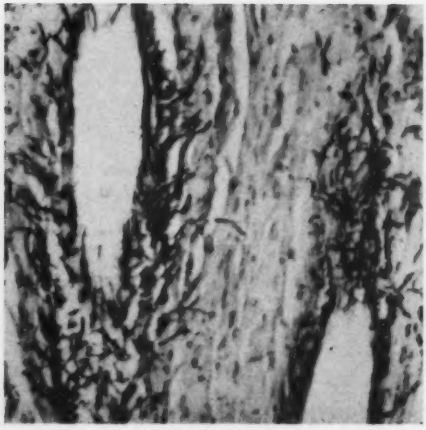

Fig. 11.

Fig. 12.

LEGENDS TO FIGURES

Fig. 7. Release of spores from a hypha after four days' growth in broth culture. Each initial hyphal septum has given rise to a mulberry-shaped cluster of spores as a result of binary fission in both transverse and longitudinal planes. (Crystal violet; magnification 1,600 x.)

Fig. 8. Stained smear prepared from an emulsified lumpy wool scab, showing a hypha at an advanced stage of internal division. Note that the cocci are all in transverse discs, one cell thick. This is evidently because confinement within the host tissue prevents binary fission in transverse planes. (Crystal violet; magnification 1,600 x.)

Fig. 9. A zoospore which has germinated and budded on a flake of the *stratum corneum* on part of a sheep's skin excised 18 hours after defatting and the application of a spore suspension. (Gram; magnification 1,600 x.)

Fig. 10. Skin fixed at 42 hours after the application of spores. Two hyphae have penetrated almost perpendicularly through the *stratum lucidum* and the underlying epidermis, which, in other sections, could be seen to be cornifying after being separated from the dermis by cellular exudate. (Gram; magnification 660 x.)

Fig. 11. Hyphae below the level of the epidermis, in follicle sheaths thickened by the accelerated cornification. This specimen was taken seven days after the application of a culture. (Gram; magnification 325 x.)

Fig. 12. Zoospores which have been released at the surface of the scab of a three-month-old lesion. (Gram; magnification 1,600 x.)

sum of three separate environmental influences: (1) restriction of air supply, (2) temperature and (3) the concentration of nutrient material of protein origin. The following experimental examples illustrate the influence of these factors on the commencement of germination.

1. Germination on the surface of agar.—For freshly isolated strains to grow when spores were spread on the agar medium, it was found necessary either to restrict the air supply by the sealing of the culture tubes, or to enrich the medium by the addition of blood or serum. It was only necessary for either of these conditions to be fulfilled, but growth became apparent sooner when both were fulfilled.

2. Germination in buffered glucose solutions containing different concentrations of peptone.—In 0·02M glucose solutions buffered with 0·005M phosphate or 0·005M barbiturate the zoospores did not germinate but continued to be actively motile for many hours, even when the air supply was restricted and the temperature was held at 37° C., which is optimal for germination. When 0·02 p.c. Difco Bacto Peptone was added to the solution, the spores were highly motile for even longer but, once again, they did not germinate. If, however, the peptone concentration was increased to 0·2 p.c., the spores commenced to germinate after several hours, even at a temperature as low as 30° C.

3. Germination at low temperatures.—When zoospores were suspended in the broth medium in sealed tubes, they germinated after a few hours at 30° C., but at 4° C. there was either delayed germination of only a few spores, or complete failure to germinate.

The process of germination was studied by immersion refractometry. Samples were taken at hourly intervals from zoospore suspensions in broth medium incubated at 37° C. The zoospores were centrifuged out of each sample, suspended in albumin solutions of different strengths and then examined by phase contrast microscopy.

Zoospores grown in liquid media had an average protoplasmic solids content of 33 p.c. After incubation for one hour, a few of them had lost their motility and had undergone a fall in protoplasmic solids content to about 27 p.c.

At the end of the second hour, a larger proportion of the population was seen to have become non-motile with a reduction of solids content. At about this time a new phenomenon appeared, an increase in size in an occasional spore. By the third hour very many had begun to enlarge and some had attained their maximum diameter. This, on an average, was double their initial diameter, so that their volume was increased by a factor of eight. During this enlargement the protoplasmic solids content remained constant, at about 27 p.c., which indicates that the spores had grown and had not simply swollen by osmosis.

Since the initial fall in solids content was associated with loss of motility and was followed immediately by growth, it may be considered as signifying a change in the nature of the protoplasm, from the denser type within the zoo-

spore whose function is locomotion, to the more watery type within those stages whose main function is growth. To this rapid change it seems appropriate to apply the term "germination". The enlarging coccus may then be considered as a hypha, even before it has budded. No evidence has been found to suggest that zoospores can fuse sexually.

Budding of the spore.

When the spores had finished enlarging, the cell wall appeared to yield at one point, where it became distended to enclose a bud. In many of the spores it was quite apparent that the wall surrounding the bud was much thinner than that surrounding the body of the cell. This suggests that the area of wall through which the bud will protrude may lose its tensile strength and be distended by osmotic pressure until it is stretched in a thin sheet over the resulting bud of protoplasm. This suggestion is supported by the fact that, at this stage, the wall sometimes yielded completely and the cell became ballooned by water intake to many times its original size, with a corresponding fall in solids content. There may be a similar mechanism to that which evidently operates in *Candida albicans*, in which rupture of the mother cell wall apparently follows the enzymic reduction of disulphide groups which bond the wall's structural components, as discussed by Rose (1960).

The buds extended during the next one or two hours until they were about 15μ long and from 0·6 to 1·0μ wide, giving the budded spore the appearance of a drumstick. By then the wall of the bud had usually increased to about the same thickness as that of the mother spore. The solids concentration had sometimes fallen by a further 1 or 2 p.c. at this stage, and was uniform throughout the protoplasm within both the old spore wall and the new bud. Moreover, there was no partition of any kind discernible, so that the whole structure appeared to be one continuous hyphal cell. Budding spores are illustrated in Fig. 2.

Hyphal growth.

After this point in the cycle, the hypha continued to extend at its growing tip. This was accompanied by a process of division by transverse septation which commenced in the oldest part of the hypha, within the old spore wall, and proceeded along the hyphal shaft at much the same speed as the extension of the hypha at its tip, so that there was always a region of new undivided protoplasm extending for a distance of from 5 to 30μ behind the growing tip. The first septum formed either within the old spore or at the point where the bud emerged, and further septa appeared at intervals of from 3 to 6μ along the hypha. The resulting segments were then divided into halves by a second wave of septation, which, like the first, passed along the hypha from the old spore toward the growing tip. Further successive waves followed, dividing the hypha into shorter and shorter segments until the septa were only from 0·3 to 0·5μ

apart. This internal division was accompanied by a steady increase in protoplasmic solids concentration, indicated by phase contrast as a darkening of the protoplasm along the hypha from the growing tip. Extension and septation of the hyphae, are illustrated in Fig. 3.

Branching of the hypha began at about the same time as the onset of septation. The first lateral bud appeared towards the spore end of the hypha, suggesting that a degree of maturity is as necessary for a region of the hypha to produce a bud as to produce a septum. Extension and internal division proceeded in each branch in the same manner and at about the same speed as in the parent hypha. A branching hypha is shown in Fig. 4.

Spore formation.

When air supply and temperature were kept fairly constant, at levels suitable for growth, the progress of the cycle depended on changes in the availability of nutrients. So long as the nutrient concentration was adequate, the hyphae continued to extend and branch. When the concentration fell, presumably below a certain critical level, i.e. after the culture had been growing for a certain time without supplementation, the second stage of growth began. Extension ceased and there was more rapid division throughout the mycelium. The formation of transverse septa in a part of the hypha ceased when the resulting segments were from 0·3 to 0·5μ in length. Each segment then grew, changing in shape from a short cylinder to a longitudinally compressed sphere as in Fig. 5. This stage is followed in the lesion, by the binary fission in planes parallel to the hyphal axis, first of the compressed sphere and then of the successive generations of its daughter cells. In this way, each segment gives rise to a number of small cocci, all of which lie in the one transverse plane, as may be seen in Fig. 8. If this continues for long enough, the hypha may be thickened 8 or 10-fold. In cultures, where hyphal extension during division was not restricted by the confines of the host tissue, there was binary fission in both planes, so that each septum gave rise to a mulberry-shaped cluster of cocci, as shown in Figs. 6, 7. Otherwise the process was similar. These cocci appeared to be somewhat analogous to the pre-swarmers of *Rhizobium* spp. They were much smaller (0·3 to 0·4μ) than the mature zoospores (0·5 to 1·1μ) and seemed to be physiologically intermediate between the hypha and the zoospore. Like the hypha they were non-motile and could divide but they had dense protoplasm and underwent the same process of germination and budding as the zoospore when the culture was supplemented by the addition of 1·0 p.c. peptone. Under these conditions the budding led to a fresh period of growth by hyphal extension.

Eventually, binary fission ceased and the third stage of growth began. The cocci increased in size, became motile and escaped. This has been seen after the first binary fission in poorer media such as peptone water, and in heavily seeded cultures, and after 8 or 9 divisions in rich media, such as V.F. broth,

and in very lightly seeded cultures, suggesting that this change in the cycle is also influenced by a fall in the nutrient concentration.

Factors which govern completion of the life cycle.

The completion of the life cycle, with the release of mature zoospores, occurred sooner at lower temperatures and with a free air supply, especially if aeration was increased by agitation in the case of liquid media. Both factors presumably raised the threshold nutrient levels below which the life cycle underwent the changes of phase just described. The changes took place earlier, before there had been heavy growth, and this ensured that adequate nutriment would remain for the conversion of the cocci to mature zoospores. Since each coccus doubles its diameter in the process, it follows that there will be an eightfold increase in the total amount of protoplasm in the culture. Therefore, an adequate nutrient supply is still very important at this late stage, even though there is no increase in the number of cells.

In broth cultures the organism has unrestricted access to the materials present so that, at higher temperatures, e.g. 37° C., the nutrient supply falls rapidly and the medium is often apparently exhausted before the third or even the second stage of growth is complete. It has been observed that the early hyphal growth is far heavier at 37° C. than at 27 or 30° C., and that further growth follows the addition of peptone to the arrested culture. This indicates that a depletion of nutrients rather than an accumulation of inhibitory metabolic products causes the premature cessation of growth.

On solid media, access of the organism to the nutrients is restricted and the supply must fall slowly as material diffuses from distant parts of the medium to the site of growth, in response to the local reduction in concentration produced by the organism's intake. Thus, on agar, even at 37° C., cocci continue to divide and mature for days, especially in single colonies remote from the regions of heaviest growth.

Factors which influence the life cycle in the lesion.

The mycelium invades the cornifying cells which form the *stratum lucidum* and which are shed in bands from the infected epidermis. The densest growth is seen, as shown in Fig. 11, where there is the greatest concentration of these cells, notably around wool follicles, against which the cornifying bands become compressed as a result of tension from exudation and exfoliation between the follicles. Bull (1929) showed that the organism digests gelatin and casein. Possible cell protein digestion is suggested in stained sections of lesions, where hyphae may be seen lying in branched spaces which often appear to cut at right angles through the cornifying cells.

The diffusion of nutrients through the tissues of the lesion may keep the food supply at a level adequate for the completion of fruiting, as postulated

for cultures on agar. In fact, however, free mature zoospores can only be found at the surface of the scab, and where there are fissures in it. All of the mycelium near the surface can be seen, in sections, to have achieved an advanced degree of division into cocci, as shown in Fig. 12. In bands of cornifying cells in the depths of chronic lesions the mycelium is often diffusely distributed but it has degenerated and has lost its affinity for dyes without having undergone more than the first stages of internal division. Presumably the nutrient supply for the organism within the successive cornifying bands is not very different from the supply near the surface of the most superficial band. Therefore, the difference is probably due to one of the other factors governing the progress of the life cycle, air supply or temperature. Because of the inflammatory hyperaemia of a lesion the underlying skin must be almost at internal body temperature and, so long as the external temperature is lower than this there will be a temperature gradient across the lesion. Therefore it would usually be warmer in the depths of the lesion and cooler at the surface. On the other hand, the surface of the lesion is more accessible to the outside air, so that both factors may be involved.

Other species of bacteria have never been detected in the depths of active lesions. The skin thus seems to provide a natural selective medium where *D. dermatonomus* can grow without competition. Invasion of the skin, growth in the lesion, and the release of spores are illustrated in Figs. 9 to 12.

The development of the capsule and its function as a spore-bearing structure.

Austwick (1958) speculated on the nature of possible structures enclosing the dividing cocci. He suggested that they might be contained within the old hyphal wall, which may be subdivided by the transverse septa into a multilocular sporangium. Thompson (1954) and Thompson and Bisset (1957), however, believed that the filament simply breaks up into cocci, and made no mention of a containing structure.

Organisms were harvested from cultures at different stages of growth and suspended in solutions containing from 30 to 35 p.c. bovine plasma albumin, both with and without a small admixture of India ink. The suspensions were then examined by phase contrast microscopy.

A thin capsule became visible just prior to the stage when the segments began to bulge, and could be seen to thicken during the rounding of the segments and their first binary fission. The capsule appeared to be fully developed by the end of the fourth longitudinal binary fission when it was wider than the bundle of dividing cells. An early stage in its formation is seen in Fig. 5. The capsules of the different hyphae remained separate in broth cultures, except within mycelial clumps or colonies. In growth on agar, and in the colonies in broth, the capsules were fused into a continuous matrix. In the growth from agar, parts of the capsular matrix had lost many or all of their cells, so that a

branched tubular system remained in the matrix. India ink particles and an occasional coccus were visible within the vacant tubes. This suggests a degree of rigidity unusual for capsular material.

Apart from the capsules, there was no structure visible which could have held the cocci together. Evidently mature zoospores break out of the capsule by means of their own motility and leave it behind as an empty tube. The capacity of the tube to hold the cells together was apparent in hyphae where some cocci were gone, leaving others held in place with spaces between.

The organism's failure to produce a sporangium may be due to the fact that it would be redundant in the lesion, where the space it occupies in the cornifying cell mass forms a relatively rigid tube. Moreover, any further enclosing structure would add to what already probably constitutes a serious problem for the relatively short-lived zoospore, the difficulty of escaping to the exterior.

DISCUSSION.

In this paper the life cycle of *Dermatophilus dermatonomus* has been described in some detail. The close interaction between environment and the growth and metamorphosis of the organism has been indicated. The physical and chemical changes taking place in the one apparently influence or even determine the development of the other. It seems clear that the requirements of *D. dermatonomus* for growth in nature must be exacting. In a nutritionally poor medium either the zoospores do not germinate or the mycelium, failing to complete its long and involved cycle of growth, dies before a fresh crop of spores has been produced. The *stratum lucidum* of the sheep's skin, on the other hand, does provide conditions suitable for the completion of the life cycle, with freedom from competition by other bacteria. As mature motile spores are released at its surface the lesion can be a direct source of spores for the establishment of fresh infection.

Since there has been no evidence to show that *D. dermatonomus* can grow anywhere else in nature and in view of the results reported here, it may be concluded for the present that the skin of the sheep is the organism's specific habitat and that the mycotic dermatitis lesion is the sole source of zoospores for transmission of the disease. It may also be concluded that, if *D. dermatonomus* does not grow outside the skin, survival of the species will completely depend on the repeated transmission of infection.

Acknowledgments. I should like to thank Dr. D. F. Stewart, Associate Chief, and Mr. J. H. Thomas, Research Officer, of the Division of Animal Health, C.S.I.R.O., for their encouragement and advice. It is a pleasure to acknowledge the valuable technical assistance provided by Mr. G. C. Merritt and Mr. I. G. Darvey, who prepared the tissue sections for photomicrography, by Mr. H. H. Offord, who prepared the bacterial culture media, by Miss L. J. Toby, who assisted me in conducting the laboratory work and by Mr. I. T. Roper, who developed and printed the photomicrographs.

REFERENCES.

Austwick, P. K. C. (1958): Vet. Rev. Annot., 4, p. 33.
Austwick, P. K. C., and Davies, E. T. (1958): Vet. Rec., 70, p. 1081.
Barer, R. (1956): In "Physical Techniques in Biological Research". Vol. 3, p. 29. Ed. G. Oster and A. W. Pollister, Academic Press, New York.
Barer, R., and Joseph, S. (1954): Quart. J. micr. Sci., 95, p. 399.
Barer, R., and Joseph, S. (1955a): *Ibid.*, 96, p. 1.
Barer, R., and Joseph, S. (1955b): *Ibid.*, 96, p. 423.
Bekker, J. G. (1928): J. S. Afr. vet. med. Ass., 1 (2), p. 51.
Bull, L. B. (1929): Austral. J. exp. Biol., 6, p. 301.
Harris, A. N. A., and Harding, W. B. (1950): Yearb. Insp. Stk. N.S.W., p. 35.
Henry, M. (1928): Pastoral Rev., 38, p. 1067.
Moule, G. R., and Sutherland, A. K. (1947): Austral. vet. J., 23, p. 95.
Roberts, D. S. (1957a): *Ibid.*, 33, p. 141.
Roberts, D. S. (1957b): *Ibid.*, 33, p. 233.
Rose, A. H. (1960): Sci. Amer., 202, p. 136.
van Saceghem, R. (1915): Bull. Soc. Pat. exot., 8, p. 354.
van Saceghem, R. (1916): *Ibid.*, 9, p. 290.
Seddon, H. R. (1929): Vet. Res. Rep. N.S.W., No. 5, p. 10.
Seddon, H. R. (1953): Serv. Publ. Dep. Hlth. Austral. vet. Hyg., No. 10, p. 434.
Simmons, G. C. (1955): Austral. vet. J., 31, p. 296.
Thompson, R. E. M. (1954): J. Path. Bact., 68, p. 445.
Thompson, R. E. M., and Bisset, K. A. (1957): Nature, Lond., 179, p. 590.

AN IMPROVED METHOD FOR ASEPTIC ULTRAFILTRATION

by J. R. THAYER AND G. C. OWEN

(From the Commonwealth Serum Laboratories, Melbourne).

(*Accepted for publication 29th August, 1961.*)

SUMMARY.

An apparatus is described which has been designed for the rapid sterile ultrafiltration of antiseptic-free biological material to give a high degree of concentration and purification.

INTRODUCTION.

In the preparation of biological materials it is often necessary to remove low molecular weight material during the purification and concentration of protein solution. The method frequently used is ultrafiltration employing collodion membranes of controlled porosity, but owing to the slow rate of filtration and the nutrient nature of certain protein solutions, such as culture filtrates, there is the problem of contamination with bacterial growth unless antiseptic is added.

The apparatus to be described provides a means of ultrafiltration in a closed sterile system through which large quantities of antiseptic-free material can be processed without contamination occurring.

MATERIALS AND METHODS.

Membrane preparation.

Hansen (1948) described the preparation of collodion membranes and this process was modified by Baxter (1953) for use with tubular refractory filters. More recently Flint and Forbes (1959) reported on the use of agar films as ultrafilters for the large-scale concentration of proteins in solution.

In the present apparatus use is made of "Corning" glass fritted tubular filters, coarse, 10-inch by 1¾-inch, which are fitted into a suitable rubber stopper along with associated fittings prior to formation of the membrane.

The membrane material is prepared from 70 p.c. cellulose nitrate in ethanol which is diluted with 99 p.c. glacial acetic acid to a final concentration of 6 p.c. cellulose nitrate. This solution is stored under vacuum (to remove air bubbles) in the dark. It has not been found necessary to remove the ethanol from the cellulose nitrate before diluting with the acetic acid. The final concentration depends on the particular batch of cellulose nitrate and on the material being ultrafiltered. We have found a final concentration of 6 p.c. to be satisfactory for tetanus toxoid and 8 p.c. for diphtheria toxoid.

To prepare the membrane the cellulose nitrate solution is carefully poured into a suitable vessel, for example, a measuring cylinder, and the clean, dry filter is immersed to the base of the stopper for two minutes. It is then removed, rotated gently in a horizontal position to give an even layer and finally held vertical to drain. During this period it is examined to ensure that there are no bubbles in the material on the filter as these usually lead to perforation of the membrane. When only single drops form at the base of the filter it is suspended completely immersed in distilled water. After two hours the filter is removed, drained and resuspended for a further twenty-four hours in fresh distilled water. After draining, the filter is fitted into the mantle, Fig. 1, to give a filter unit ready for sterilization, care being taken that the membrane is not touched or damaged.

Sterilization of the filter unit.

The filter unit is sterilized by immersion, with complete filling, in a 1 p.c. phenol solution in a suitable single filter unit container, for a minimum period of seven days. It is our practice to store prepared filter units in this solution until required for use. When they are handled carefully these filters have a life of many months and at the completion of an ultrafiltration they can be rinsed with distilled water, without dismantling, and returned to the phenol solution.

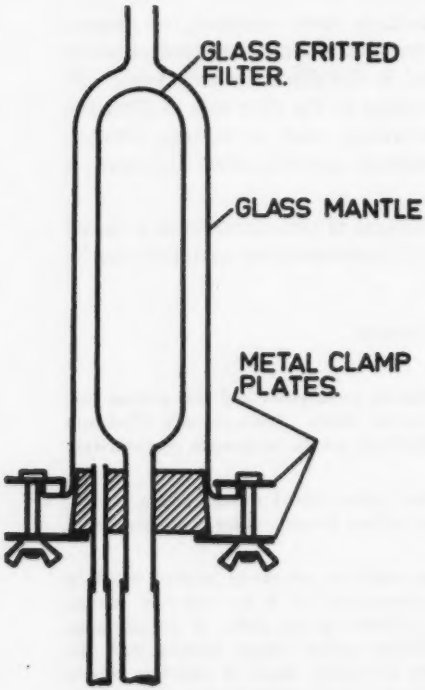

Fig. 1. Filter unit.

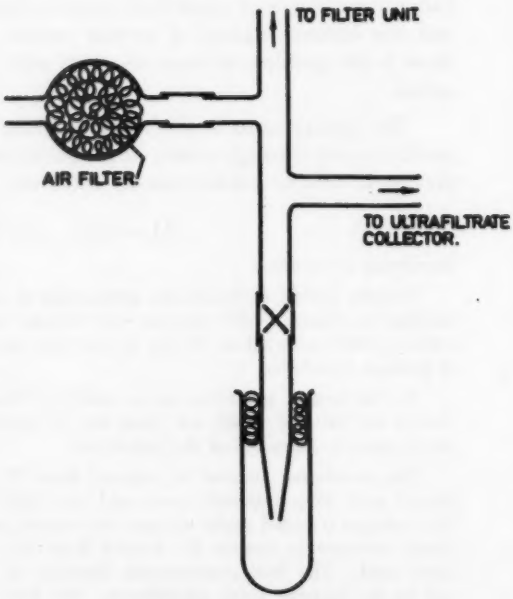

Fig. 2. Sampling unit.

Assembly of the apparatus.

The filter unit is removed with sterile forceps from the phenol solution and fitted into the rest of the apparatus which has been previously sterilized by autoclaving. The filter is freed of phenol by the filtration of a small quantity of sterile distilled water. The sampling unit, Fig. 2, which is necessary for periodical testing of the ultrafiltrate to ensure that there is no breakdown of the membrane, is constructed from stainless steel tubing to eliminate the possibility of breakage during sampling (see under Operation). For the ultrafiltration of large volumes of material a series of filter units can be used in parallel as indicated in Fig. 3. Under normal conditions we use one filter unit for each thirty litres of material to be ultrafiltered.

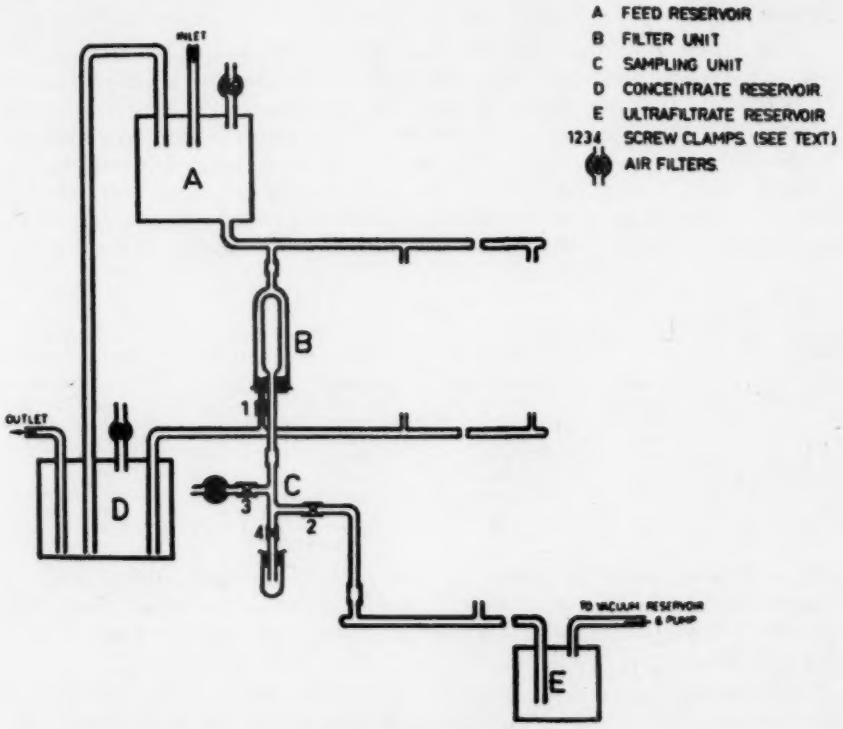

Fig. 3. Assembled apparatus.

OPERATION.

Air-locks, which could lead to the drying out of the membrane with subsequent damage, are removed by allowing sterile distilled water to run by gravity from the feed reservoir through the filter mantle to the concentrate reservoir by opening screw-clamp 1. It is then returned back along the same path by the

application of air pressure to the concentrate reservoir until the filter unit is full of liquid and none remains in the reservoir. The screw-clamp 1 is then closed. The vacuum pump is now switched on and is controlled by a pressure-sensitive switch to maintain a minimum vacuum of 25 inches of mercury in the vacuum reservoir. When only a small volume of water remains in the feed reservoir, the material to be ultrafiltered is added.

When a sample of the ultrafiltrate is required, clamp 2 is closed to isolate the membrane filter from the vacuum line. Clamp 3 is then slowly opened to allow the entry of air through the sterile cotton wool filter and the sample which has collected in the lower end of the unit can be obtained by opening clamp 4.

During the operation of the ultrafilter the concentrated material which collects in the mantle around the filter can usually be detected by its different colour or refraction. It is periodically drained into the concentrate reservoir, usually twice daily. If required, the concentrate can be returned after concentration to the feed reservoir to which sterile distilled water or saline has been added. After thorough mixing, repetition of the ultrafiltration is carried out. By this procedure salts can be removed or a greater degree of purity obtained. As shown in Table 1, greater than fiftyfold concentration has been readily obtained in the

TABLE 1.

Concentration of tetanus toxoid by ultrafiltration.

Batch	Initial Lf./ml.	Ultrafiltrate Lf./ml.	Residue Lf./ml.	Residue Lf./mg.N	No. of times concentrated
80	20	No. flocc.	2100	700	105
81	26	No. flocc.	1400	760	54
82	20	No. flocc.	1400	760	70
83	20	No. flocc.	1200	700	60

case of tetanus toxoid in culture medium. The ultrafiltration of a batch is ceased at a time convenient for further processing and the values obtained indicate the final concentrations and not the maximum which can be obtained with this apparatus.

After a few hundreds of litres have passed through the filter, the rate of filtration may be lowered to such an extent that the membrane has to be renewed. The old membrane is peeled off and the glass fritted filter is cleaned with chromic acid in the normal way. It can then be re-coated as described previously.

REFERENCES.

Baxter, E. H. (1953): Australasian J. med. Tech., 2, p. 19.
Flint, G. A., and Forbes, O. C. (1959): Manufacturing Chemist, 30, p. 493.
Hansen, Albert (1948): Acta path. microbiol. Scand., 25, p. 460.